THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XIII AUGUST, 1932 No. 4

CHOLESTEROL VARIATIONS IN EXPERIMENTAL ANÆMIAS.

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Received for publication April 28th, 1932.

IT has been known for some considerable time that variations in the cholesterol content of the blood occur in different types of anæmia. Thus Erben (1902) observed that the cholesterol content both of the corpuscles and plasma was diminished in cases of chlorosis. Again Pacini (1918) found a marked deficiency in the serum cholesterol in cases of pernicious anæmia, and recorded improvement in this disease by the administration of cholesterol through lanoline inunction. Kipp (1920), Denis (1917), Bloor and Knudson (1917), Bloor and McPherson (1917), MacAdam and Shiskin (1923), and others have also made observations in clinical varieties of anæmia which in general show the blood cholesterol values to be low. It is also noted that as a rule the decrease is more marked in the plasma than in the corpuscles.

The present writers have made similar observations in clinical cases confirming the findings of the authors mentioned, but as the results add nothing new, they are not recorded here.

Whilst hypocholesterolæmia in anæmias is the rule, it is worthy of note that Feigl (1921) found hypercholesterolæmia as a part of a lipæmia in cases due to hæmorrhage.

Very few observations have been made in experimentally produced anæmias. Boggs and Morris (1909) recorded the production of an intense lipæmia in rabbits by bleeding the animal repeatedly, but no observations were made on the cholesterol content of the blood; indeed the technique for accurate estimation of cholesterol had not been developed at that time. No similar lipæmia was caused when the anæmia was produced by pyrodin.

Sakai (1914) confirmed the observations of Boggs and Morris, and found that hypercholesterolæmia was an accompaniment of the lipæmia, a finding corroborated by Horiuchi (1920) and Bloor (1921).

By the work recorded here the writers hoped to throw further light on the variations in blood cholesterol in anæmias produced experimentally, and to

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supply some information (at present lacking), as to the variation in the cholesterol content of the tissues.

EXPERIMENTAL.

Buck rabbits were used for all experiments, and were kept in the laboratory for several weeks before use, on a diet of bran, oats and greens.

Anæmia was produced: (*a*) by bleeding in varying amounts, (*b*) by subcutaneous injection of phenylhydrazine hydrochloride. A third method was tried, namely, intravenous injections of sodium oleate, but owing to the high mortality and the difficulty of avoiding local inflammatory changes in the ear, it was discarded. One experiment, more successful than the others, is, however, included here. Bleeding was performed by puncture of the peripheral vein of the rabbit's ear, and amounts varying between 10 and 50 c.c. were withdrawn, once or repeatedly.

Profound anæmia was produced by subcutaneous injections of phenyl hydrazine hydrochloride in solutions of varying concentration. After early failure in the choice of a suitable dose it was found that a 1 per cent. solution gave the best results. Full details of the amount of blood withdrawn or the amount of phenylhydrazine administered will be gathered from the various tables.

For the estimation of cholesterol in whole blood or plasma, the method of Myers and Wardell (1918) was used with slight modifications as made by MacAdam and Shiskin (1923). The principal additional modification introduced by the writers was to evaporate the whole 25 c.c. of chloroform extract until it reached a volume of about 6 c.c., and to make the colorimetric estimation on this. This has the advantage of giving a deeper colour with the Liebermann-Burchard reaction, and thereby facilitates the colorimetric readings.

For the same reason the writers used 2 c.c. of blood or plasma instead of 1 c.c. as originally described by Myers and Wardell. This was necessary owing to the cholesterol content of rabbits' blood being lower than that of man. In a group of 17 control experiments where the cholesterol was estimated by this method, the whole blood showed a maximum content of 151 mgm. per cent., and a minimum of 61, whilst the plasma gave a maximum figure of 88 and a minimum of 20. The average was 97 mgm. per cent. for whole blood and 47 for plasma. The cholesterol of tissues was estimated by Grigaut's (1911) method. In the case of small organs, such as the spleen and suprarenals, the whole organ was used for the estimation, but in the case of larger organs, such as the liver, it was necessary to mince the organ and take a mixed sample.

All animals rendered anæmic were allowed ample fluids and their normal diet between the bleedings or injections.

Except when a profound anæmia was produced rapidly, the animals showed little disturbance of health.

Hæmoglobin estimations were made by the Haldane-Gower hæmoglobinometer and the blood volume was calculated from Dreyer's formula $B = W\ 2/3\ K$, where B = blood volume in cubic centimetres, W = weight of rabbit in grammes, K = constant varying with each species of animal, in rabbits 1·58 (Dreyer and Ray, 1911).

Lipæmia was graded roughly according to the degree of opalescence, a faint degree being styled ½+ whilst ++++ represents a serum resembling milk.

RESULTS.

These may be considered under three headings according to the manner in which the anæmia was produced.

A. *Anæmia Produced by Hæmorrhage.*

Most of the experiments fall into this series, and a further subdivision is necessary in order to analyse the results more easily. An arbitrary division into the following groups has been made:

1. Repeated large hæmorrhages; Experiments 1, 2, 3, 4, 13, 17.
2. Large single hæmorrhages; Experiments 5, 6, 7, 10.
3. Repeated small hæmorrhages; Experiments 8, 9, 11, 12, 14.

TABLE I.—*The Effects of Repeated Large Hæmorrhages. Note the fall of Suprarenal Cholesterol Content at Height of Lipæmia in Experiment* 13.

Rabbit No.	Day of experiment.	Body weight (gm.).	Blood volume (c.c.).	Amount of bleeding (c.c.).	Character of plasma.	Cholesterol. Whole blood mgm. per 100 c.c.	Plasma mgm. per. 100 c.c.	Suprarenals gm. per 100 gm.	Spleen mgm. per 100 gm.
4	1	2500	117	30	Clear	89	42	..	..
	2	..	..	30	Lipæmia ½+		Not estimated.		
	3	..	..	30	Lipæmia +	73	35	..	..
	4	..	..	28	Lipæmia +++	..	31	..	..
	5	..	..	35	Lipæmia ++++	130	119	..	..
	6	..	..	30	Lipæmia ++++		Not estimated.		
	7	..	..	*Nil*	..	..	..	..	..
	8	..	..	30	Lipæmia ++++		Not estimated.		
	9	..	..	*Nil*	..	..	..	..	..
	10	..	..	23	Lipæmia ++	156	179	..	..
	11	..	..	*Nil*	..	..	..	4·9	279

Rabbit No.	Day of experiment.	Body weight (gm.).	Blood volume (c.c.).	Amount of bleeding (c.c.).	Character of plasma.	Cholesterol. Whole blood mgm. (per cent.).	Plasma mgm. (per cent.).	Suprarenals gm. (per cent.).	Liver mgm. (per cent.).
13	1	2550	118	35	Clear	128	65	..	..
	2	..	..	35	Lipæmia ½+		Not estimated.		
	3	..	..	35	Lipæmia ++	123	59	..	..
	4	2550	..	35	Lipæmia +		Not estimated.		
	5	..	..	38	Lipæmia +++	120	59	..	..
	6	2450	..	30	Lipæmia ++++	192	155	2·2	310

Group I. Repeated large hæmorrhages.—In this group of experiments from 20 to 30 c.c. of blood were withdrawn daily. There was a fall in the cholesterol content both of the whole blood and of the plasma on the 2nd and 3rd days of the experiment, and in the majority of cases (Experiments 2 and 17 excepted) the fall was more marked in the plasma than in the whole blood. In most instances a slight lipæmia was obvious as early as the 2nd day, but increased greatly on the 3rd and 4th days, and usually continued until the end of the experiment. About the 4th day, that is, 24 to 48 hours after the appearance of the lipæmia, the blood cholesterol regained its original pre-hæmorrhage values, whilst on the 5th, 6th and subsequent days, very high figures were reached for the cholesterol content of blood and plasma. The cholesterol content of the plasma showed the greatest changes, in one experiment showing an increase of more than 400 per cent. over the original value (Experiment. 4).

It is also to be noted that in several experiments, when the animal was purposely killed at the height of the lipæmia, the cholesterol content of the suprarenals was abnormally low (Experiments 1, 13 and 17 ; see also Tables I and VI).

Experiment 2 was irregular in many ways, and no conclusions can be drawn from it.

TABLE II.—*Changes during the Course of a Single Large Hæmorrhage (Experiment 7).*

Day of experiment.	Body weight (gm.).	Blood volume (c.c.).	Amount of bleeding (c.c.).	Character of plasma.	Cholesterol. Bleeding (c.c.).	Whole blood mgm. per 100 c.c.	Plasma mgm. per 100 c.c.	Suprarenals gm. per 100 gm.	Spleen mgm. per 100 gm.
1	2220	107	57	Clear	First 10	87	37	..	..
					27–37	79	31	..	..
					47–57	61	23	0·95	290

Group II. Single large hæmorrhages.—In this group the animal was bled once only, to the extent of about 50 c.c. Generally the animals stood the hæmorrhage well, but one died with convulsions.

Portions of the blood were taken at the beginning and end (sometimes also in the middle) of the bleeding, and were examined for their cholesterol content.

The figures show that, in most cases, between the beginning and end of the hæmorrhage there was a definite fall in the cholesterol content of the whole blood and plasma.

With the exception of Experiment 7, the suprarenal cholesterol content remained within average normal limits, and it is doubtful if any significance should be attached to this exception, as occasional extreme variations from the normal are found in the suprarenal cholesterol content of normal animals.

No lipæmia of significant grade occurred after a single large hæmorrhage.

Group III. Repeated small hæmorrhages.—In this group the animal was bled in approximate amounts of 10–15 c.c. daily, or on alternate days, for 7–28 days. In two experiments (Experiments 8 and 9) an initial large bleeding was also made.

In none of these experiments was there a significant lipæmia. Slight

TABLE III.—*Effects of Repeated Small Hæmorrhages* (*Experiment* 12).

Rabbit No.	Day of experiment.	Body weight (gm.).	Blood volume (c.c.).	Amount of bleeding (c.c.).	Character of plasma.	Cholesterol. Whole blood mgm. per 100 c.c.	Cholesterol. Plasma mgm. per 100 c.c.	Cholesterol. Suprarenals gm. per 100 gm.	Cholesterol. Spleen mgm. per 100 gm.
12	1	2005	100	14	Clear	97	74	..	..
	2	..	..	17	,,	85	47	..	..
	3	..	..	15	,,		Not estimated.		
	4	..	..	17	,,	..	37	..	..
	5	..	..	12	,,	89	36	..	..
	6	..	..	17	,,	76	29	..	..
	7	..	..	*Nil*	..	..	..	..	..
	8	..	..	15	Clear	..	30	..	..
	9	..	..	18	,,	65	37	..	..
	10	..	..	15	,,	67	33	..	..
	11	..	..	15½	,,		Not estimated.		
	12	..	..	2	,,	58	..	..	..
	13	..	..	*Nil*	..	..	..	3·7	440

grades were sometimes observed, but never of the degree seen after repeated large hæmorrhages.

The blood cholesterol content decreased steadily in most experiments, but corresponding with the absence of the lipæmia there was an absence of any secondary rise in the cholesterol content of the plasma, such as is observed after repeated large hæmorrhages.

The suprarenal cholesterol content was variable, in one experiment (Experiment 8) being above the average, in the others below (Experiments 9, 11 and 12). No conclusions can be drawn from these figures as they are within the limits of physiological variation.

To sum up, it is to be noted that in all three groups there is the same tendency for an initial fall followed by a secondary rise to occur in the blood and plasma cholesterol, but as would be expected, the grade of variation depends upon the extent of the hæmorrhage.

Tissue Cholesterol Content.

The variations in the cholesterol content of the suprarenals when the animal was killed at the height of its lipæmia have already been noted.

Inspection of Table VI will show that, comparing 17 animals rendered anæmic by bleeding, with 20 normal animals, there is a decrease in the average cholesterol percentage in the adrenals from the normal 7·3 to 5·3, whilst the total amount of cholesterol in the organs divided by the body-weight is 15 mgm. in the normal, and 11 mgm. in the anæmic rabbits.

There is on an average, therefore, less chloesterol in the suprarenals after bleeding, in spite of the fact that many experiments are included where no marked lipæmia occurred.

The only other tissues examined were the liver and spleen, the cholesterol content of which does not show any change outside the limits of physiological variation. In control experiments the average tissue cholesterol content was as follows:

Suprarenals	7·3 gm. per 100 gm.
Spleen	380 mgm. ,, ,,
Liver	285 ,, ,, ,,

TABLE IV.—*Effects of Sodium Oleate.*

Day of experiments.	Body weight (gm.).	Amount of bleeding (c.c.).	Character of plasma.	Hæmoglobin per cent.	Cholesterol. Whole blood mgm. per 100 c.c.	Cholesterol. Plasma mgm. per 100 c.c.	General condition of rabbit.	Amount of sodium oleate injected.
1	1950	8	Slight hæmolysis	70	105	35	..	5 c.c. 2 per cent. solution.
2	..	..	..	..	..	..	Quite well	No injection.
3	..	8	Slight opalescence. Hæmolysis +	56	83	33	..	Unable to inject owing to thrombosis.
4	..	..	..	..	..	..	..	Injected 1·5 c.c. 5 per cent.
5, 6	..	..	..	..	..	..	..	No interference.
7	..	8	Hæmolysis +	50	98	52	..	..
8	..	..	..	..	..	..	No ill effects	4 c.c. 5 per cent.
9	..	7	Hæmolysis ++	46	86	54	..	..
10	..	..	..	..	..	..	..	No interference.
11	..	..	..	..	..	..	..	3 c.c. 5 per cent. solution.
12, 13	..	..	..	..	..	..	..	No interference.
14	..	..	..	..	..	..	..	4 c.c. 5 per cent. solution.
15	..	5	..	45	106	34	..	..

B. *Anæmia Produced by Sodium Oleate Injections.*

Although a considerable number of experiments was attempted, the success of this method of producing anæmia was very limited, and only one experiment deserves inclusion here. (Table IV.)

In this the animal was injected with a 5 per cent. solution of sodium oleate (intravenously) at intervals of 2 to 4 days, over a period of 15 days. Marked hæmolysis was produced, but the animal remained in good health until it was killed on the 15th day. The hæmoglobin was reduced from an initial value of 70 to 46 per cent., and during the whole experiment there was no lipæmia, although slight grades of this might have been masked by the hæmolysis.

The variations in the blood cholesterol content will be seen in the table, but no conclusions can be drawn from them beyond noting the general tendency of the cholesterol values to fall, followed by a slight secondary rise.

C. *Anæmia Produced by Phenylhydrazine Hydrochloride.*

A number of experiments was made with this powerful hæmolytic agent, but owing to unsuitable dosage the animals died. The results of four successful experiments, however, are available. In the first, doses of 1 c.c. of a 5 per cent. solution of phenylhydrazine were given and a profound hæmolysis occurred, reducing the hæmoglobin content of the blood from 60 to 15 per

cent. in three days. It was not possible to determine the presence or absence of slight grades of lipæmia owing to the dark-red appearance of the plasma.

There was, however, a significant change in the plasma cholesterol content which rose from 40 to 104 mgm. per 100 c.c. within three days. The cholesterol content of the whole blood was not estimated owing to insufficient amounts of blood being available.

In the second experiment small repeated doses of phenylhydrazine were given and in a period of twelve days the hæmoglobin was reduced from 80 to 45 per cent. In spite of this no material change took place in the cholesterol content of the blood. Possibly the maintenance of normal figures for the blood and plasma cholesterol throughout the experiment, indicates that the usual initial fall was quickly compensated by the secondary rise (*vide* Experiments 3 and 4), but that this rise was not sufficient to bring the figures much above their original values.

TABLE V.—*Effects of Phenylhydrazine* (*Experiment* 3).

Day of experiment.	Weight of rabbit (gm.).	Amount of bleeding (c.c.).	Character of plasma.	Hæmoglobin per cent.	Cholesterol. Whole blood mgm. per cent.	Cholesterol. Plasma mgm. per cent.	Cholesterol. Liver mgm. per cent.	Cholesterol. Adrenals gm. per cent.	General condition of rabbit.	Amount of phenylhydrazine.
1	1200	10	Slight opalescence	65	107	31	..	..	Good	1 c.c., 1 per cent.
2	..	..	..	..	..	..	..	..	..	1 ,, ,, ,,
3	..	..	..	..	..	..	..	..	..	1 ,, ,, ,,
4	..	..	..	34	..	..	..	..	..	2 ,, ,, ,,
5	..	..	..	..	..	..	..	..	Thin and ill	1 ,, ,, ,,
6	..	..	..	..	..	..	..	..	..	None.
7	..	10	Lipæmia ++	25	197	220	350	10·5	Killed	..

It is noteworthy that the animal remained in comparatively good health throughout the experiment, and lived for three weeks after the injections were discontinued.

The third experiment (Table V) showed the effect of small doses of phenylhydrazine given over a period of seven days. The hæmoglobin was reduced from 65 to 25 per cent., and a moderate degree of lipæmia (++) occurred. The variations of cholesterol in the whole blood and plasma were similar to those obtained in anæmias produced by severe hæmorrhage. There was a rise in the whole blood cholesterol content from 107 to 197 mgm. per 100 c.c., and in the plasma cholesterol from 31 to 220 mgm. per 100 c.c. (an increase of over 700 per cent.).

The last experiment (Experiment 4) in which larger doses of phenylhydrazine were given over a longer period of time (19 days) did not show such profound changes in the blood cholesterol content, though the same tendency to rise was to be observed, as in Experiment 3. In partial explanation of the slighter changes it is to be noted that the original cholesterol values were abnormally high.

The cholesterol content of the suprarenal glands in these phenylhydrazine

experiments was variable, and no conclusions can be drawn owing to the small number of experiments and the irregularity of the results. Further, no attempt was made to kill the animal at the height of the lipæmia, as in the bleeding experiments, so it was not possible to say whether there is a depletion of the suprarenal cholesterol content.

DISCUSSION.

In whatever way the anæmia was produced in the present experiments, variations in the blood and plasma cholesterol content took the same form, namely:

1. An initial fall.
2. A secondary rise.

The Initial Fall.

In the case of anæmia produced by bleeding, it seems reasonable to suppose that the fall in the cholesterol content of the blood and plasma is due, at least in part, to blood dilution, which occurs soon after any hæmorrhage.

Whether blood dilution is *entirely* responsible for the fall in cholesterol values is uncertain, and in view of the relatively crude methods of estimating cholesterol and blood volume, it is doubtful whether accurate information could be obtained on this point by comparing the decrease in the blood cholesterol with the degree of blood dilution. That blood dilution is the principal factor, however, is supported by the decrease in cholesterol values which take place even whilst the animal is *being* bled (see Group II experiments).

In these it is scarcely possible that any other mechanism, such for example as an alteration in storage or synthesis of cholesterol, could have time to come into play.

In the anæmias produced by phenylhydrazine the decrease in cholesterol must be explained in a different way, for there is not necessarily any alteration in the blood volume. It may be supposed that the disruption of the red corpuscles by the hæmolytic effects of the drug would lead to an increased amount of cholesterol in the plasma, cholesterol perhaps of no immediate value to the body, so that mechanisms of storage or excretion would be called into play to remove it, and the cholesterol values of the blood would soon fall below normal.

The figures in Group II experiments show such a decrease in the cholesterol values for whole blood, but unfortunately, the plasma cholesterol values available do not show whether any change took place in them.

The Secondary Rise.

The increase in cholesterol seems essentially to be part of a general lipæmia, and the values are higher in the plasma. The degree of hypercholesterolæmia, like that of the lipæmia, is proportionate to the severity of the anæmia, occurring to the greatest extent in those experiments when the anæmia was produced by repeated large hæmorrahges, and in one of the phenylhydrazine experiments (Experiment 3). The maximum intensity of the hypercholesterolæmia

is considerably later than that of the lipæmia (1 to 4 days). This accords with the observations of Bloor (1921), who found that in lipæmia, fat increased first, then lecithin and finally cholesterol.

In respect of these cases of hypercholesterolæmia, two difficult problems must now be discussed :

(*a*) Whence does the increase of cholesterol come ?

(*b*) What is the purpose of this increase ?

The *origin of the cholesterol* cannot be determined with any degree of certainty, for our present knowledge of the physiology and pathology of cholesterol is very limited.

Many authorities are of the opinion that the cholesterol of the body is principally, or entirely, derived from exogenous sources—the food. Others, especially the French school as represented by Chauffard, Laroche and Grigaut (1918), incline to the view that at least part of the cholesterol may be supplied to the body from the synthetic activities of the suprarenal glands.

One of us (Chamberlain, 1928) has brought forward evidence to support the latter view, but it seems reasonable to suppose that the suprarenals may have a double function in relation to cholesterol, viz., synthesis and storage.

Whatever view may be held as to the exact relationship between the suprarenals and cholesterol, there seems little doubt that these glands are more likely than any other tissue to be concerned in cholesterol metabolism.

It was without surprise, therefore, that when the suprarenal cholesterol content was specially examined at the height of lipæmia, it was found to be abnormally low in many cases. That this state of affairs did not hold good in every case has little significance, for the great normal variations in the suprarenal cholesterol content is well known, and figures below or above the average 7 per cent. are often found.

The depletion of the suprarenals at the time when the blood cholesterol is abnormally high suggests that some of the cholesterol in the suprarenals has been transferred to the blood. As the glands can store between 9 and 40 mgm. of cholesterol, they could furnish an appreciable portion, though probably not all, of the increase of cholesterol in the blood. Thus, in Experiment 13, out of a total increase of 106 mgm. of cholesterol, 23 mgm. could be accounted for from suprarenals (*vide* Experiment 13 and Table VI). The remainder may come from other tissues (though there is no indication from our investigations that any other tissues are participating in the cholesterol changes), but more probably the amount of cholesterol shown to be present in the suprarenals at the time of death represents only a portion of the amount available from this source, for if the glands have synthetic activities they may continually produce small amounts of cholesterol which are at once passed into the blood.

The Purpose of the Increase.

The significance attaching to the lipæmia in general and to the hypercholesterolæmia in particular, remains also largely a matter for speculation.

It is known that the cholesterol possesses considerable antihæmolytic powers against saponin, cobra venom, tetanolysin, etc. (Ransom, 1901 ; Fraser and Gardner, 1909 ; Simon *et al.*, 1909 ; Noguchi, 1903).

TABLE VI.—*Cholesterol Content of the Suprarenals following Hæmorrhage.*

Rabbit No.	Weight in gm.	Weight of suprarenals in gm.	Suprarenal tissue per kilo-body weight in gm.	Percentage of cholesterol in gm.	Total cholesterol in mgm.	Total cholesterol divided by body-weight in kilos.
1	2120	0·56	0·264	1·8	10	5
2	2010	0·615	0·306	6·15	38	19
3	2190	0·54	0·246	7·4	40	18
4	2500	0·44	0·176	4·9	22	9
5	1740	0·48	0·276	6·8	33	19
6	2030	0·30	0·148	9·6	29	14
7	2220	0·415	0·187	0·95	4	2
8	2150	0·40	0·186	9·2	37	17
9	2020	0·445	0·220	3·5	16	8
10	1880	0·320	0·170	9·4	30	16
11	2530	0·335	0·132	5·6	19	7
12	2005	0·570	0·284	3·7	21	11
13	2550	0·475	0·186	2·2	10	4
17	2360	0·747	0·317	2·4	18	8
Average			0·221	5·3	23	11
Average in 20 controls			0·219	7·3	27	15

It is also known that these antihæmolytic powers depend to a great extent on a correct cholesterol-lecithin ratio (Brinkman and Van Dam, 1920). In various forms of lipæmia it has been shown that there is a disturbance of the cholesterol-lecithin ratio, a disturbance which may have existed in the present experiments when marked lipæmia was found.

In order to maintain the normal cholesterol-lecithin ratio, the body may (through the suprarenals) produce an increased amount of cholesterol, even an excessive amount ; but in experiments where hypocholesterolæmia occurred and persisted, it is possible that the degree and rapidity of production of the anæmia was not sufficiently great to evoke this protective hypercholesterolæmia. In a disease like pernicious anæmia in which hæmolytic agencies are thought to be at work, the low cholesterol values may serve to keep up the hæmolytic processes.

The clinical experiments of Pacini (1918) are not very convincing in showing the expected benefits of cholesterol administration in pernicious anæmia, but it is notoriously difficult to increase the blood cholesterol content in any way, and even if low cholesterol values occur in pernicious anæmia, this could hardly be supposed to be *wholly* responsible for the disease.

On the other hand it is interesting to note that Beck (1928) has observed marked increases in the blood cholesterol content in anæmias of various types (especially pernicious anæmia) which have responded well to liver treatment, and has even been able to increase simultaneously the red corpuscles and cholesterol content of the blood of normal people by means of liver diets.

The last observation is of particular interest in view of the difficulty previously experienced in producing an alimentary hypercholesterolæmia except in herbivorous animals.

In short, the theory may be advanced that the hypercholesterolæmia which follows hypocholesterolæmia in certain cases of anæmia is protective in nature,

designed to overcome the serious hæmolytic effects of a deficiency of cholesterol in the blood. This deficiency may be due to a reduction in the number of red corpuscles with their contained cholesterol, as for example in cases of hæmolysis, or to a dilution of the plasma as in cases of hæmorrhage.

SUMMARY.

1. Experiments were made to observe the changes in the cholesterol content of the blood and other tissues in cases of anæmia. The anæmia was produced experimentally in two main ways: (*a*) by bleeding, (*b*) by subcutaneous injections of phenylhydrazine hydrochloride.

2. In anæmias produced by bleeding, the following results were obtained:

(*a*) In nearly all cases a preliminary fall occurred in the blood and plasma cholesterol values. It is thought that this was due chiefly to blood dilution.

(*b*) In cases where the bleeding was made in large amounts over several days, this preliminary fall was followed by a secondary rise, in which the cholesterol values both of the blood and plasma attained figures several hundred per cent. above the normal. These increases were a part of a general lipæmia.

(*c*) When the animal was killed whilst a marked lipæmia was present, the cholesterol content of the suprarenals was usually low, and it is suggested that the suprarenals had been depleted to supply part of the increased amount of cholesterol in the blood.

(*d*) The reason for the hypercholesterolæmia is not obvious, but as cholesterol possesses notable anti-hæmolytic properties, it may be a protective phenomenon to prevent the evil effects of the initial fall in blood cholesterol.

(*e*) In the case of small repeated hæmorrhages, or large single hæmorrhages, the primary hypocholesterolæmia occurred and persisted, but there was as a rule no secondary rise in cholesterol values.

3. The injection of sodium oleate to produce anæmia was unsuccessful except in one experiment, in which there was a slight primary hypocholesterolæmia, followed by a slight hypercholesterolæmia later. The figures obtained were not at all decisive.

4. The variations in blood and plasma cholesterol values in anæmias produced by phenylhydrazine were similar to those found in cases of hæmorrhage, *i. e.*, there was an initial fall followed by a secondary rise, illustrating that hypercholesterolæmia in anæmias is not peculiar to the anæmia of hæmorrhage.

We would like to acknowledge the help and advice that we have had from Prof. Macdonald, in whose department this work has been carried out, and from Prof. Channon.

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THE GROWTH OF *B. PYOCYANEUS* IN SYNTHETIC MEDIA.

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Received for publication May 1st, 1932.

I. AËROBIC GROWTH.

The minimal necessary pabulum for aërobic growth has been found here to consist of nitrogen, organic carbon, magnesium and phosphorus dissolved in suitable form and concentrations at pH 6–9 in distilled water. The following three formulæ give satisfactory growths: (1) Citric acid 0·5 gm., ammonium hydrate (·880) 0·5 c.c., saturated magnesium hydrogen phosphate solution to 100 c.c.; (2) sodium lactate 0·5 gm., ammonium dihydrogen phosphate 0·2 per cent. solution, neutralized by excess magnesium oxide and filtered, to 100 c.c.; (3) succinic acid 0·5 per cent. solution, neutralized by excess magnesium ammonium phosphate and filtered. These quantities include water of crystallization.

(a) *Experimental Details.*

Test-tubes used to grow the organism were Monax, $6 \times \frac{5}{8}$ in., plugged with wool and cleaned by dichromate-sulphuric mixture.

Incubation at 37° C.

Growth was observed macroscopically and judged by opacity.

The inoculum was one drop (approximately 0·025 c.c.) of a suspension consisting of two drops of synthetic culture in 10 c.c. distilled water. The synthetic cultures were at least eight generations removed from an agar slope. They were incubated for two days and were used until one month old, during which time they were kept at room temperature.

(b) *Strains.*

Three strains of *B. pyocyaneus* have been used: "Greaves" (London Hospital), "Fildes" (N.C.T.C. 1999) and "Bart.'s" (N.C.T.C. 2005). Although "Fildes" was inferior in growth and dye production to the other two, no qualitative differences were observed between the three strains, nor any alterations in the character of each.

(c) *Essential Substances.*

Phosphorus.

This element has been used in these experiments only in the form of phosphate, either as mineral salt or with an organic radicle (ethyl phosphate, sodium glycerophosphate). No visible growths have ever been obtained in its absence, though Butterworth and Walker (1929) omitted it from their medium.

Magnesium.

Experiments conducted with re-crystallized salts have shown that growth does not usually occur in absence of this element. Moreover, if to a medium lacking magnesium is added a salt which has not been purified (*e.g.* calcium chloride), slight growths may occur, whilst the addition of the same anion prepared so as to exclude magnesium (calcium sulphate is suitable) is without effect. Nevertheless shreds of growth and slight opalescences are seen sometimes (particularly in media containing mineral acids) in absence of magnesium.

Nitrogen and carbon.

Availability means here the liability of a foodstuff to be metabolized by the organism. In the case of phosphorus and magnesium the element appears to be available in whatever form it is present, but nitrogen and carbon are available in some substances and not in others. Availability is to be distinguished from *the degree of nourishing power* possessed by different available foodstuffs. Thus the combination in a medium of a poorly nourishing but available carbon compound (*e.g.* ethyl alcohol) with a poorly nourishing but available nitrogen compound (*e.g.* sodium nitrate) may fail to show visible growth, and lead to the false conclusion that neither foodstuff is available.

Nitrogen has been found available in ammonium salts, nitrates and amino-acids; but not in nitrites, urea, mono-ethylamine, aniline, picric acid, betaine, guanidine or pyocyanin (prepared from synthetic cultures).

Carbon has been found available in carbamate, acetate, lactate, pyruvate, succinate, butyrate, citrate, glycerol, glucose, amino-acids, ethyl alcohol and normal butyl alcohol; but not in carbonate, urea, acetaldehyde, acetone,

normal propyl alcohol, guanidine, glycerophosphate, betaine, many sugars and aromatic compounds, including pyocyanin.

A suitable basal medium for testing amino-acids was found to be saturated magnesium hydrogen phosphate solution. For carbon sources in general, autoclaved tubes of distilled water with a pinch of magnesium ammonium phosphate at the bottom were often used. The solid does not interfere with the readings, since the liquid above it is clear and the pH of the liquid (about 8) is not altered by addition of acids. Indeed, by digesting excess of the salt with an organic acid till the required pH is reached, filtering, diluting and sterilizing, media may be produced in which the organism will grow and make pigment in the absence of the usual adventitious ions (*e. g.* sodium, etc.).

No visible growth has ever been obtained on carbonate in these experiments. Braun (1921) found opalescence in carbonate media after a week, but suggests (1922) that his inocula contained enough bacterial bodies and metabolites to serve as food for such growths. Such an explanation of the growth of the organism on carbonate is unsatisfactory, in that it may be propagated indefinitely from tube to tube of carbonate medium without any visible growth appearing in any tube at any stage, its presence being shown by subculture to agar. Table I illustrates the propagation of the organism on various media.

TABLE I.

Medium.	Viability on daily subculture.						
	1.	2.	3.	4.	5.	7.	10.
0·1 per cent. $(NH_4)_2CO_3.H_2O$ in aq dest.	+	+	+	+	+	+	+
0·5 per cent. ditto	+	+	+	0	..	..	..
0·1 per cent. $NaHCO_3$ in aq. dest.	+	+	+	+	+	+	+
0·1 per cent. NH_4Cl in aq. dest	0	..	..	..	..	..	..
0·1 per cent. NH_4Cl in M/20 KH_2PO_4 buffer pH 7·8	+	+	+	+	+	+	+
Excess $MgNH_4PO_4.6H_2O$ in aq. dest.	+	+	+	+	+	+	+
Aq. dest.	0	..	..	..	..	..	..
Tap-water	+	+	+	+	+	+	+
Saline	0	..	..	..	..	..	..

+ indicates absence of visible growth, but growth on subculture to agar.

All salts were recrystallized. A loop of clean platinum was used for subculture, which was performed every day from one tube to the next and to an agar slope. Carbonate media were unsterilized, controls being incubated and subcultured to agar without growth. No differences were observed when the carbonate media were sterilized by filtration, when a glass pipette (cleaned by dichromate-sulphuric mixture) was used instead of the loop, when wool plugs were eliminated, or when water distilled from alkaline permanganate was substituted for ordinary distilled water.

The propagation in magnesium ammonium phosphate and in buffer solutions, in spite of precautions to exclude every trace of carbon, suggested that carbon dioxide, absorbed by alkaline fluids from the air, was responsible for growth. This was shown to be the case in the following way :

Magnesium ammonium phosphate was prepared by precipitation, washed quickly and repeatedly with boiling distilled water, and distributed to test-tubes as a suspension in boiling distilled water. The test-tubes were then

plugged with glass-wool and cooled quickly. One tube was inoculated, and all placed in an anaërobic jar containing also tubes of 40 per cent. potassium hydroxide. The jar was exhausted and allowed to refill with air drawn through soda-lime, potash and baryta (the latter demonstrating absence of carbon dioxide). The jar was opened once a day for subculture and refilled. After four subcultures the organism showed diminished growth (on the agar indicator); after six, the slope showed only a few discrete colonies ; after eight, there was no growth. Propagation continued indefinitely in a control jar containing ordinary air.

In these growths it must be carbonate which is the limiting factor. Yet no variations in concentrations, nor the expedient of bubbling carbon dioxide through a magnesium-ammonium phosphate culture, have produced visible growth. Such a paradox, namely, that a substance can initiate growth yet not continue to support it on a basal medium otherwise adequate, seems only explicable on the hypothesis that the organism is deriving its carbon from carbamate, which co-exists in solution with carbonate according to the equilibrium :

$$2\ NH_2COOR + 2\ H_2O = (NH_4)_2CO_3 + R_2CO_3.$$

Fenton (1885) showed that on solution of ammonium carbamate in water equilibrium is reached in three hours at a concentration of about 10 per cent. carbamate to 90 per cent. carbonate. Thus in a solution of 0·1 per cent. ammonium carbonate (concentrations above this are inhibitory), the amount of available carbamate offered to the organism is 0·01 per cent. This is the minimal concentration value determined here for two carbon compounds. Friedlein (1928) gives the same minimal figure for glucose. Hence, allowing for continuous bacteriolysis, conditions for subminimal growth appear to be realized. Moreover, the organism has been propagated (subminimally) in ammonium acetate solution too dilute to show visible growth.

For the subminimal growths described in this experiment it is possible that no magnesium or phosphorus are necessary, or that the minute amounts required are present as impurities ; but the source of nitrogen, in the case of those media which do not contain it, has not been found.

The propagation of *B. pyocyaneus* in tubes of autoclaved tap-water served, on one occasion, to isolate the organism from peritoneal pus containing a mixture of bacteria.

(d) *Ions Liable to Affect Growth.*

It was found that alteration in growth could occur on addition to synthetic media of a variety of ions, such as sodium, potassium, calcium, chloride, sulphate or carbonate. If media are constructed in which these ions are excluded in turn growth is not visibly affected. The organism also grows well on magnesium ammonium phosphate digests containing none of them (*supra*), and growth on such digests is not improved by adding these ions. The conclusion is therefore drawn that they are not specifically necessary to the growth of the organism. On the other hand, the following experiment may be quoted : If, in addition to sodium citrate and magnesium sulphate, the following three solutions are employed alternatively as basal media :

(1) 0·25 per cent. KH_2PO_4 and 0·1 per cent. NH_4Cl,
(2) 0·22 per cent. $NH_4H_2PO_4$ and 0·13 per cent. KCl,
(3) 0·22 per cent. $NH_4H_2PO_4$,

the equality of the results with (1) and (2) and the poorer growth using (3) leave no doubt that some function is fulfilled by the potassium chloride. But addition of this salt to other media may result in diminished or unchanged growth according to the constitution of the medium used. The explanation is therefore suggested that these ions act by displacing ionic equilibria in the media.

The extreme chemical purity necessary to deal accurately with these ions has not been attempted here.

(e) *Concentrations.*

The concentrations of substances in synthetic media required for growth of *B. pyocyaneus*, *B. coli* and *B. paratyphosus B* formed the subject of a paper by Friedlein (1928), and fairly similar minimal values for *B. pyocyaneus* have been obtained here, though it has been found that foodstuffs possess minimal and optimal concentration ranges rather than definite values.

Before quantitative determinations could be made on the foodstuffs by varying them independently in a medium, it was necessary to establish their independent variability. Preliminary experiments of simultaneous variation of constituents in a medium were therefore undertaken, of which Fig. 1 illustrates the type. They indicated no special necessary ratios between ions (*e.g.* that ammonium should be ten times as concentrated as phosphate), but rather that a constant basal active mass of each substance is required independently of variations in others, whether growth is aërobic or anaërobic.

In most cases the optimal ranges of concentration are wide for aërobic growth, whilst those for anaërobic and pigmentary activity tend to be more limited regions within these ranges. In addition to a definite minimal concentration value, the existence of a minimal zone just above this value is shown by the occurrence within it of non-pigmented growths. Table II shows the optimal and minimal concentration ranges of various foodstuffs as found in these experiments :

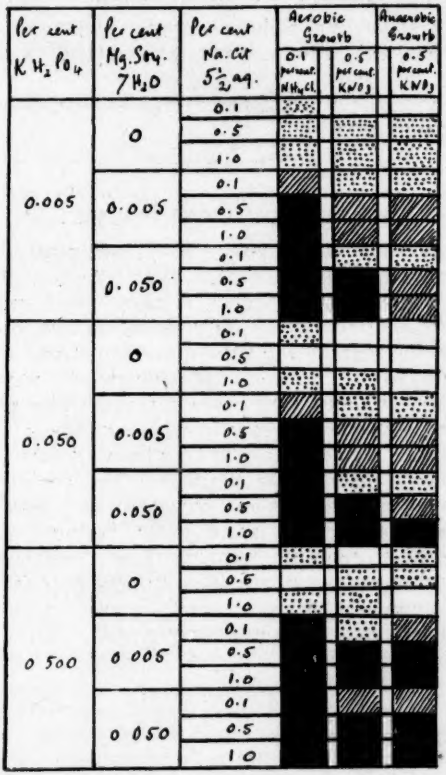
TABLE II.

	Minimal range (per cent.).	Optimal range (per cent.).	Anaërobic optimum (per cent.).	Pigmentary optimum (per cent.).
Na, K, Cl, SO_4	0	0	0	0
$MgSO_4$, $7H_2O$	0·0001–0·0005	0·0005–0·5000	0·05	0·05
KH_2PO_4	0·0010–0·0025	0·0025–2·0000	0·50	0·05
NH_4Cl	0·0025–0·0500	0·0500–0·2500	..	0·10
$NaNO_3$	0·0100	0·1000–2·0000	0·50	..
Na citrate, $5\frac{1}{2}$ aq.	0·0100–0·2500	0·2500–2·0000	1·00	1·00
Na acetate, 3 aq.	0·0100–0·0500	0·1000–0·2500	0·10	0·25

II. ANAEROBIC GROWTH.

Few particular and no generalized formulæ have been found in the literature for synthetic media capable of growing the organism anaërobically. In these

experiments the availability of foodstuffs has been found the same as for aërobic growth, but their nourishing power is decreased by the anaërobic conditions, so that absence of growth is more likely to occur. However, it has been possible to cultivate the organism anaërobically on any of the available



Results of one week's growth of " Greaves " on a medium in which the concentrations of all constituents except the nitrogen source were simultaneously varied. Each tube of medium was individually titrated to pH 6·8 by soda or hydrochloric acid. All salts were recrystallized. No growths were obtained in that part of the experiment (not shown in Fig. 1) where the phosphate concentration was zero. Intensity of shading indicates degree of growth.

carbon compounds by addition of nitrate only to the medium (as sole source of nitrogen), and, in the case of poorly nourishing substances, by manipulating concentrations. This adjustment consists (*cf.* Table II) in increasing the phosphate concentration up to or beyond the upper limit of the aërobic optimal range; sometimes, also, a decrease in the concentration of the carbon compound may assist growth.

The following formula provides an efficient anaërobic medium (water of

crystallization included): Sodium citrate 1·0 gm., sodium nitrate 0·5 gm., magnesium sulphate 0·05 gm., M/20 phosphate buffer (pH 7·6) 100 c.c.

Oxalates have provided the only anomaly in that, in this work, they have supported growth anaërobically, but not aërobically. Braun (1921), however, achieved aërobic growth on oxalate.

Other substances containing a nitrogen-oxygen linkage (sodium nitrite, picric acid, choline and betaine) failed to replace nitrate as oxygen source for the anaërobic growth of this organism, but Quastel (1925) found sodium nitrite, and glucose slightly, efficacious.

The occurrence of traces of pigment in anaërobic tubes has been found the index of inefficient deoxygenation.

The cultures have been incubated throughout in an anaërobic jar.

III. NOTE ON PIGMENTATION.

Aubel (1913) demonstrated the presence of both pyocyanin and "fluorescein" in synthetic media, but noted that their production was "subject to caprice." Results here obtained show (1) that the formation of both its pigments by *B. pyocyaneus* requires only those substances necessary for its growth, but (2) that the pigmentary function is sensitive to two factors which are of minor relevance to the function of aërobic growth, namely (*a*) the degree of atmospheric oxidation, and (*b*) concentrations. The yield of pyocyanin from synthetic cultures of various constitutions has never been found to equal that from bacto-peptone water; on the other hand, "fluorescein" production can apparently exceed that in the peptone. The surface/volume ratio of cultures forms a convenient measure of oxygenation. The following formula provides a medium suitable for pigmentation: Sodium citrate ($5\frac{1}{2}$ aq.) 1·0, calcium sulphate 0·025, magnesium sulphate (7 aq.) 0·05, ammonium chloride 0·1, potassium dihydrogen phosphate 0·2 per cent., in 0·25 per cent. ethyl alcohol; surface/volume = 0·25.

A high phosphate concentration inhibits pigment.

IV. DISCUSSION.

1. *Essential substances.*—Phosphorus, carbon and nitrogen are generally accepted as necessities for the growth of *B. pyocyaneus*, whilst some doubt is thrown on magnesium; Braun (1921) omitted it from his medium. But no previous statement has been found of the constancy of these necessities for different conditions and media. The strongest argument obtained here for the necessity of magnesium is its possession of a minimal concentration range in which non-pigmented growths occur, similarly to the other necessary foodstuffs.

2. *Availability.*—This property appears to be an absolute one. Although the ethyl alcohol-nitrate combination does not grow the organism when the medium is neutral, it can be made to do so by addition of alkali. This eliminates the alternative explanation that there is a specific relation between alcohol and ammonium (a growth-promoting combination). No theory of specific interdependence of substances, but rather the constancy of behaviour of the organism towards all, has been the finding in these experiments.

SUMMARY.

The requirements of *B. pyocyaneus* (3 strains) for minimal growth and for pigment production as determined on many permutations of synthetic media under various conditions are constantly four (phosphorus and magnesium with available nitrogen and carbon). The only special case observed is the necessity of nitrate for anaërobic growth. The behaviour of the organism on these media is described.

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THE ACTION OF BETA RADIATIONS FROM RADIUM ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO.

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Received for publication May 17th, 1932.

Two sets of experiments were carried out. In the first, a radium applicator containing 60 mgm. radium, over an area of 4 sq. cm., with 0·12 silver screenage, was strapped on to the egg-shell to one side of the embryo, for periods of time varying from 1/4 to 68 hours.

In the second, a window about 1/2 sq. cm. in area was cut in the shell by means of the technique described (Goulston and Mottram, 1932), and the applicator affixed over the window.

It follows that the second set of experiments only differed from the first in that the egg-shell was not present between the radium and the allantoic membrane.

Before being applied to the shell window, the radium applicator was scrubbed with soap and water, rinsed with sterile water, and placed in 80 per cent. alcohol, then thoroughly dried with a sterile towel. A piece of lead of the same size similarly treated was employed in the case of the controls. The allantoic membrane was thus radiated through the shell membrane for periods varying from 1/4 to 2 hours. All the experimental eggs were placed in separate sterile tins placed in the incubator during radiation. After radiation the

applicators were removed, and in the case of eggs with windows, were replaced by pieces of shell, as in the previous paper.

In all cases the eggs were incubated for a period of three days after the commencement of radiation, before being opened for examination.

Twenty-two eggs were used in the first experiment and 10 eggs in the second experiment, and 11 served as control specimens to the second set. No

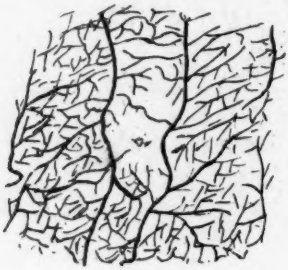

FIG. 1.

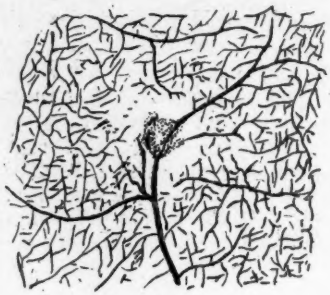

FIG. 2.

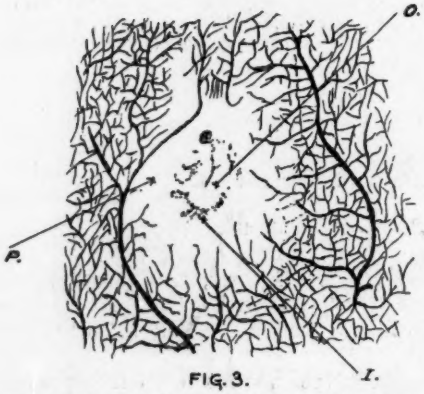

FIG. 3.

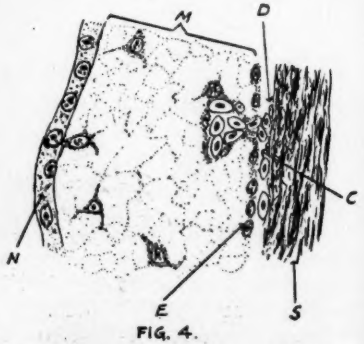

FIG. 4.

FIGS. 1–3.—Natural size drawings of the allantoic membrane of three specimens radiated without windows, Fig. 1 for 1/2 hour, Fig. 2 for 1 hour and Fig. 3 for 2 hours. The membranes were fixed in Bouin's solution. O = central dead area; I = narrow area of irregular hypertrophy; P = peripheral region showing early degeneration.

FIG. 4.— × 450. Normal allantoic membrane on the 13th day. N = endoderm; E = ectoderm; C = capillary; S = shell membrane; M = mesoderm; D = endothelial cell.

macroscopic nor microscopic reactions were observed with 1/4 hour's exposure, either with or without the shell; whilst with 1/2 hour's radiation a pale anæmic area was seen macroscopically (Fig. 1).

Increased exposure gave an increase in the size of this area, devoid of blood-vessels, as seen in Fig. 2, where a specimen was exposed for 1 hour, and in Fig. 3, where the exposure was increased to 2 hours. In the centre of this

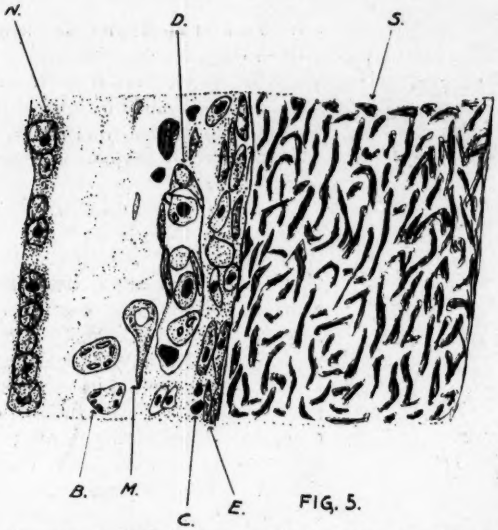

FIG. 5.

FIG. 5.—Drawing of section × 1000 showing the dead portion, o in Fig. 3, of a specimen radiated through a shell window for 2 hours. S = shell membrane; E = ectoderm and endothelial cells; D = swollen endothelial cell in capillary wall; M = swollen vacuolated mesodermal cell; B = very degenerate red cell; N = swollen degenerate endodermal cells; C = chromatin debris.

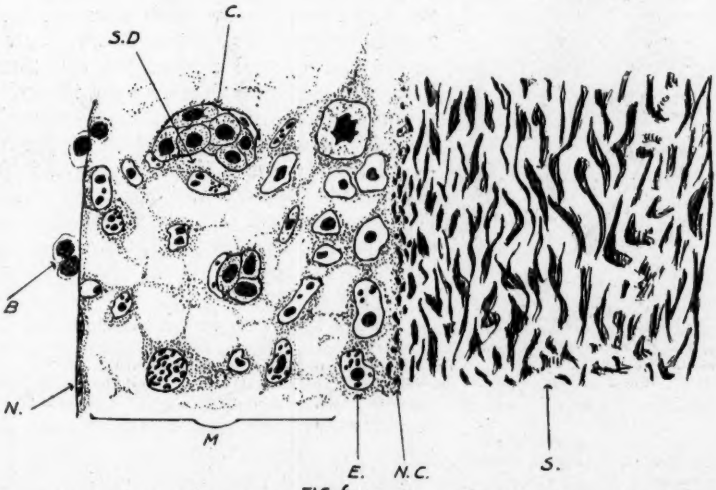

FIG. 6.

FIG. 6.—Drawing of section × 1000, taken from the central portion, o in Fig. 3, of the specimen, radiated for 2 hours without a window. S = shell membrane; N.C. = necrosed material representing egg-shell capillary; E = enlarged degenerate ectodermal cell; C = thrombosed blood-vessel; S.D. = swollen degenerated endothelial cell; M = mesodermal layer; N = endoderm; B = nuclei of degenerate corpuscles.

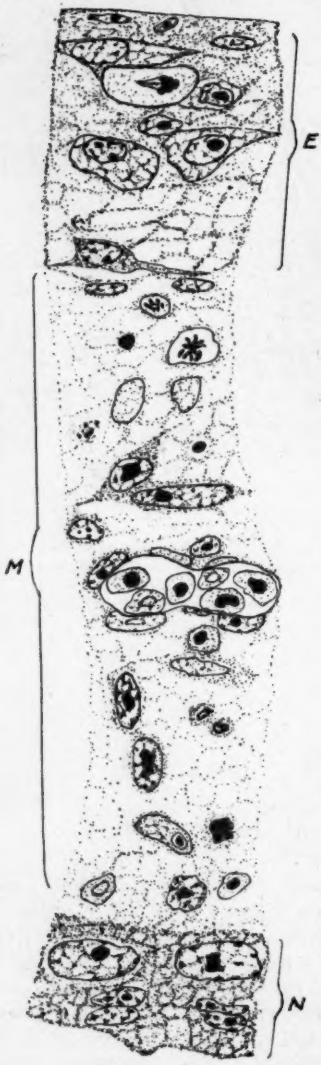

FIG. 7.

FIG. 7.—Drawing of a section of specimen × 1000, at I in Fig. 3, showing a degenerated hypertrophied area around the central dead area. The shell membrane is omitted. E = hypertrophied ectoderm with very large degenerate cells; M = hypertrophied mesoderm formed of swollen degenerate cells; N = hypertrophied endoderm with degenerate swollen cells.

anæmic area were usually seen scattered dark patches which, on microscopical examination, were found to consist of thrombosed vessels.

The histological details of the normal membrane are shown in Fig. 4, and have been described in a previous paper (Goulston and Mottram, 1932).

A microscopic examination of the reaction of the membrane after exposure to β-radiation, with or without a shell window, showed that there is (*a*) a central dead area, O in Fig. 3, surrounded by (*b*) a narrow area of irregular hypertrophy, I in Fig. 3, and (*c*) a peripheral region, showing early degeneration, P in Fig. 3.

(*a*) In the central area (Figs. 5 and 6) the shell capillary was absent, only degenerate endothelial cells remaining; the ectodermal cells were swollen and degenerate. Some thrombosed capillaries containing altered red cells and some extravasated altered red cells were seen in the mesoderm. The mesodermal cells were themselves swollen and degenerate. The cells comprising the endodermal layer were also degenerated, in some places being very swollen.

(*b*) In the narrow area of irregular hypertrophy (Fig. 7) the shell capillary was absent, as in the central region, the ectoderm was hypertrophied and degenerated, the cells containing swollen nuclei. The mesoderm showed a slight hypertrophy and some degeneration; blood-vessels were thrombosed and full of altered red cells. The endoderm was very degenerated, and in some places showed considerable hypertrophy, the cells being approximately seven layers deep, instead of the normal single layer.

(*c*) In the peripheral region (Fig. 8) the shell capillary was absent, and only degenerate endothelial cells remained. The ectoderm cells showed degeneration, and sometimes slight proliferation. Some blood-vessels in the mesoderm were thrombosed, while others contained no corpuscles. The mesoderm and endoderm showed early degeneration. When the membrane was radiated for periods longer than 2 hours, the central dead area increased in size and finally corresponded to the area of the applicator.

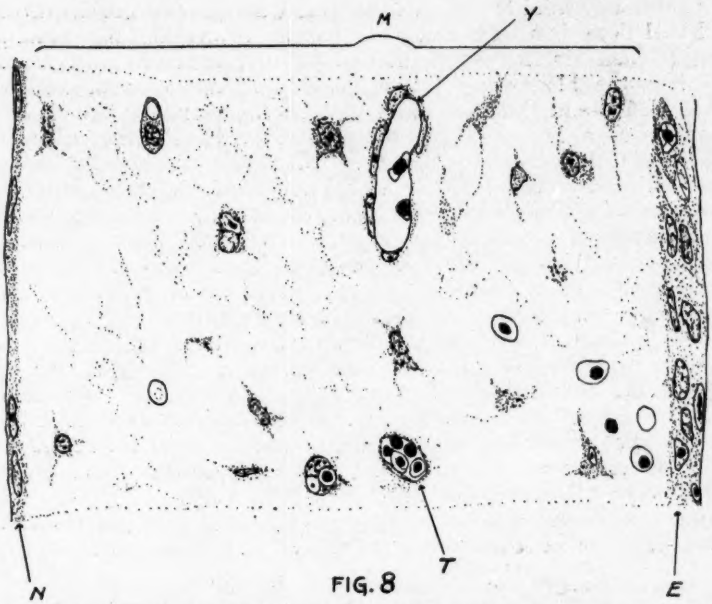

FIG. 8

FIG. 8.—Drawing of section × 1000 taken from the peripheral region of specimen (P in Fig. 3). E = ectodermal and endodermal cells, shell capillary absent; M = mesoderm; T = thrombosed blood-vessels containing some nuclei of degenerated corpuscles; Y = blood-vessel almost free of corpuscles; N = endoderm.

DISCUSSION.

The results show that the chorio-allantoic membrane is readily destroyed when subjected to β-radiation. The most striking effect of exposure was upon the blood-vessels of the membrane; even after 1/2 hour's radiation, the capillary under the shell was no longer present. The capillaries and small blood-vessels in the mesoderm in the centre of the radiated area contain only odd degenerated red cells. The endothelial cells lining the capillaries have swollen nuclei towards the periphery; the mesoderm capillaries are filled with red cells, and are thrombosed. As regards the cellular elements, in the central region there is degeneration of cells without hypertrophy, and, passing towards the periphery, is found degeneration associated with hypertrophy of all three layers; this hypertrophy must have occurred soon after radiation, since only a few abnormal mitoses were present.

Whether or no all the effects of radiation can be attributed to the action on blood-vessels, the results show that this action is of great importance. In the central region of radiation the cellular elements are entirely destroyed, showing degenerative changes, and here one can assume that there has been direct action on the cell, as well as upon the blood-supply. Away from the centre hypertrophic reaction occurred, showing that the radiation has not

been immediately fatal to the cells; evidently they had not received an immediate lethal dose, but were able subsequently to divide. Abnormal mitotic figures are found in this region three days after exposure (Fig. 7). In this region, however, blood-vessels have not escaped, since thrombosis regularly occurred. It would therefore seem that the hypertrophy was a secondary reaction to this, as has already been described (1932), following injury without radiation. Further, at the periphery of the radiated region the change was confined to blood-vessels, especially to the shell capillary—so much so that the cells of the membrane were little changed. This, therefore, indicates how especially sensitive to radiation are capillaries and small blood-vessels.

Moppett (1932) has published the results of exposing the allantoic membrane to β-rays derived from a radium D deposit. The details given of the amount used do not enable us to compare his dosage with ours. On referring to his tabulated findings, it is evident that very irregular results were obtained. Thirty-four experimental eggs were used, of which 11 died, and 20 controls, of which 6 died; of the 23 experimental eggs which survived, 5 showed " slight reaction " and 3 an " intense reaction " ; of the 14 control eggs which survived, 3 showed " slight reaction " and 1 " intense reaction." It is therefore evident that the controls were not satisfactory, and it appears that the radiated eggs did not behave differently from them.

In our experiments, positive results were always obtained above a minimal dose of β-radiation, and invariably the control showed no reaction.

CONCLUSION.

Exposure to beta-radiation for 1/2 hour from a 60 mgm. radium applicator produced degenerative and hypertrophic reactions, accompanied by profound changes in the blood-vessels in the chorio-allantoic membrane of the chick embryo.

APPENDIX.

Side by side with these observations using β-radiation, some exposures to gamma and X-radiation were made.

Gamma radiation.—An attempt was made to repeat some results obtained in Australia (Goulston, 1931). Three specimens were radiated through shell windows, using the technique described in this paper, with a 6·25 mgm. radium tube, active length 1 cm., screenage 0·5 Pt for a period of 3 to 4 hours. No reaction was obtained. Also one specimen radiated with two of these radium tubes gave negative results.

It is therefore concluded that the reactions previously obtained with radium tubes were due to exposure of eggs under non-sterile conditions. Negative results were also obtained when two eggs without a shell window were radiated with 60 mgm. radium applicator of 2 sq. cm. area, screenage 0·12 Ag plus 3 mm. Pb, for 26 hours.

X-radiation.—An attempt was made to repeat Moppett's (1925) results with X-radiation of wave-length 0·5 A°. A Seemann spectrometer was used with a Coolidge tube working at 150 volts and 3 milliamps. Two eggs were exposed for 3/4 and 1 hour, with negative results. This is not in keeping with

Moppett's results, but the number of exposures were too small to be of value.

In this connection attention may be drawn to the work of Packard (1931), who finds that the mortality curve for eggs of *Drosophila* is dependent upon the number of Rœntgen units delivered, and not upon the wave-length. Except that Packard did not use a spectrometer, this is not in keeping with Moppett's results. Until this difference is explained, Moppett's results can only be accepted with reservation.

My grateful thanks are due to Dr. Mottram for his most helpful advice and criticism while this research was being undertaken, and to Prof. Russ and Mr. Clark for the loan of the Seemann spectrometer and for the setting up of the apparatus. The radium used was on loan from the Medical Research Council.

This work was carried out under a grant from the British Empire Cancer Campaign.

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MUCOUS CHANGES IN THE EPITHELIAL CELLS OF THE RAT'S VAGINA DURING THE EARLY STAGES OF PREGNANCY.

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Received for publication May 30th, 1932.

THE vaginal epithelium of the rat undergoes a remarkable evolution during pregnancy, a fact which has been known for a considerable period. Briefly, a change occurs from a stratified squamous epithelium, producing keratin, to a stratified epithelium with mucus-containing cells. The grosser histological changes have been described by Lataste, Morau (1889), and others, and a short historical review is given by Kelly (1929). These changes are now being used as a test for the potency of corpus luteum extracts.

The present paper describes work which had as its object the observation of the finer cytological changes involved in the initial stages of this cellular transformation—observations which have not, apparently, been made as yet.

Methods.—Either the actual time of copulation with plug formation was noted, or the rats were observed twice a day and those showing plugs segregated. The animals were killed at intervals after copulation.

* With a grant from the Medical Research Council.

Histological technique.—For studying the mucin, as first formed, fixation in Zenker-formol has given good results. The granular form of the mucin is retained, and these granules take muci-carmine very intensely. In fact, mucin, both from these and from the goblet-cells of the gut stains intensely after this fixative—rather better, usually, than after fixatives such as formol or "Susa."

The mitochondria were fixed in Nassonov's modification of Champy's fluid and stained by Heidenhain's iron-hæmatoxylin.

The Golgi bodies were impregnated by Cajal's alcoholic uranium nitrate mixture. Fixation for 6–7 hours and then 36 hours in 1·5 per cent. silver nitrate gave the best results. After this fixative the nuclei stained excellently in Delafield's hæmatoxylin and the mucus took the muci-carmine intensely.

OBSERVATIONS.

The epithelium of the vagina of a rat in œstrus consists of several layers of cells, those nearest the lumen producing droplets of eleidin. The surface is covered with layers of keratin. Both these substances stain intensely with iron-hæmatoxylin.

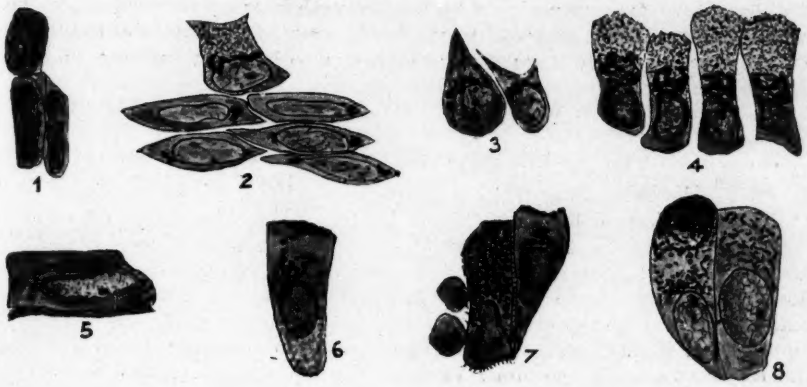
After impregnation the superficial keratin layer and cells showing eleidin droplets disappear, and at the same time there is considerable mitotic activity in the basal layers of the epithelium. About the third day stainable mucus begins to appear in cells of the crypts, and to a lesser degree in some cells abutting on the lumen, while at the same time cells of other parts of the vagina may exhibit eleidin and keratin formation—a fact noted by Robson and Wiesner (1931) in a study of the effects of injecting extracts of corpus luteum. The mucus appears first of all near the nucleus, between it and the vaginal lumen. The amount gradually increases as the droplets migrate towards the upper pole of the cell (Figs. 6 and 7) till the cell is full. Cells which have the nucleus oriented parallel to the basement membrane show such granules (Fig. 5).

These cells gradually increase their protoplasmic content and elongate perpendicularly to the basement membrane, and also probably revolve somewhat on their axes. A suggestion of the former is shown in Fig. 5; it is more pronounced in the uppermost cell of Fig. 2.

With the growth of the cellular protoplasm the nucleus alters in shape, becoming round or oval, with long axis perpendicular to the basement membrane. At the same time as this is taking place it comes to occupy the basal part of the cell.

The cells are then of different shapes and sizes, depending on compression exerted by their neighbours. By 8 days after impregnation, only the deepest layers of epithelium exhibit no mucus, the rest of the cells being stuffed with it. At the same time the nucleus is compressed to one side. After good fixation and suitable staining, intercellular protoplasmic bridges are seen to exist between cells producing mucus. This is easily determinable in the early stages, but when the cells become very full of mucus they cannot be recognized (Figs. 5 and 7).

The discharge of mucus appears to go on from an early period, for stainable mucus is present in the vaginal lumen very soon after its appearance in the



DESCRIPTION OF FIGURES.

[All figures drawn using a Zeiss 2 mm. apochromat and an 8 × compensating ocular.]

FIG. 1.—Rat, 4 days pregnant. Cajal technique for Golgi bodies. Nuclei stained by Delafield's hæmatoxylin. Cells from basal layer of epithelium show small argentophile masses at side of nucleus.

FIG. 2.—Rat, same specimen as 1. Shows elongated cells with argentophile material at end of nuclei, and a mucus-producing cell with Golgi body on lumen side of nucleus, which is still longitudinally orientated. Mucus stained by muci-carmine. The contrast between the red of the mucus and black of the argentophile material is very sharp in the original preparations.

FIG. 3.—Rat, same specimen as 1 and 2. Mucus-producing cells from middle layer of epithelium to show Golgi material.

FIG. 4.—Rat, 7 days pregnant. Cells from surface layer showing elongation, nuclei at base, large amount of Golgi substance, and protoplasm above the nucleus crammed with mucus.

FIG. 5.—Rat, 3 days pregnant. Fixed Zenker-formol. Stained iron-hæmatoxylin, muci-carmine and metanil yellow. Shows a cell oriented with nucleus parallel to basement membrane of mucosa beginning to manufacture mucus droplets in relationship to lumen side of nucleus. The intercellular protoplasmic bridges are also shown.

FIG. 6.—Rat, 4 days pregnant. Same fixation and staining as previous cell. Shows development of mucus in a cell oriented perpendicularly to the basement membrane.

FIG. 7.—Rat, same as Fig. 6. Shows one cell full of granules, still preserving protoplasmic intercellular bridges, the other cell just beginning to develop mucus. Two leucocytes are represented penetrating the mucosa.

FIG. 8.—Rat, 3 days pregnant. Fixed in Nassonov's fluid. Stained Heidenhain's iron-hæmatoxylin and muci-carmine. The pale grey granules in the cell on the left are mucus (bright red in original); the dark filaments are mitochondria. This drawing shows the disposition of the elements in a young mucus-producing cell and one in which mucus has not yet been produced.

cells. The amount of mucus in the lumen increases as the mucosa becomes more " mucified." The mucous cells apparently finally disintegrate, liberating all their mucus, and are replaced by fresh growth from below. In many places cysts of mucus caused by breakdown of cells occur in the depths of the mucosa. Numerous polymorph leucocytes are always to be seen making their way through the epithelium to the vaginal lumen.

Alterations in the Golgi Bodies.

It is relatively difficult to impregnate anything of the nature of Golgi bodies in the deepest layers of the epithelium. The cells of these layers have very little protoplasm, and therefore can accommodate very little Golgi substance. Fig. 1 represents a constant finding of argentophile material, closely approximated to the nuclei in successful preparations. Fig. 2 shows similar material at the ends of the nuclei of flattened elongated cells. The topmost cell of this figure shows an increase in the amount of Golgi substance, and its orientation on the lumen side of the nucleus, which still has its long axis parallel to the basement membrane. This cell contains a considerable quantity of mucus. Fig. 3 shows two cells from the middle portions of the epithelial layer which exhibit a considerable development of argentophile material.

Fig. 4 represents four cells taken from the outermost layer of the vaginal mucosa. They contain considerable mucus, nuclei at the bases of the cells, and a very considerable volume of Golgi material. The Golgi bodies of these cells impregnate easily, and these results have been obtained on several animals.

In Figs. 6 and 7 the initial formation of mucus appears to be taking place in the area occupied by the Golgi bodies. In Fig. 5 this is not quite so clear, but comparison with the uppermost cell in Fig. 2 makes it probable that here, too, the droplets are appearing in close topographical relationship to the Golgi apparatus.

Mitochondria.

These were stained black by Heidenhain's iron-hæmatoxylin, and at the same time the mucin was stained red by muci-carmine. It is found in this tissue that the muci-carmine does not always stain the mucus after fixation in Nassonov's fluid, the reason for this being obscure. In successful preparations, showing early mucus formation, it is easy to see that all cells of the mucosa possess an abundant supply of filamentous mitochondria, rather remarkable for their variety of contorted shapes. In the deeper layers, in cells with a small amount of protoplasm, they are arranged symmetrically about the nucleus. With the increase in protoplasmic content of the cells there is an increase in the number of mitochondria present. In Fig. 8 two cells are represented, one of which contains a considerable volume of mucin granules, and at the same time a large number of filamentous mitochondria which do not appear to have any special arrangement. The cell next to it is not producing mucus, and contains many mitochondria irregularly disposed. In some cells abutting on the lumen, both those mucus-producing and those not, there is often a concentration of mitochondria beneath the free border of the cell, a

small clear area intervening between them and the border. In these early stages of mucin production it has not been possible to observe or deduce that the mitochondria are involved in this process.

DISCUSSION AND SUMMARY.

The changes exhibited by the rat vagina were investigated in the hope that additional data would be forthcoming for the understanding of pathological mucous changes—" mucous degeneration." Under the influence of some hormone (corpus luteum ?, see Mirskaia and Wiesner, 1930, and Robson and Wiesner, 1931, for a discussion of extract effects) the cellular metabolism is changed, so that instead of manufacturing eleidin the mucosa elaborates mucin. In its earliest manifestations this change of metabolism occurs with very little change in cellular form. Later the changes in form described occur. By following the initial stages it is clear that the mucification of the cells is not accomplished by a wholesale alteration of protoplasm into mucus—a " degeneration." The droplets are manufactured in that part of the cell occupied by the Golgi body in a way shown to hold true for other glandular epithelium (see Florey, 1932).

As the mucus cells develop, there is a very considerable hypertrophy of both the protoplasm and Golgi substance. This increase of Golgi substance during the elaboration of secretion has been shown to be true of some secreting glands (*e. g.* pancreas, salivary gland). Basing the argument on these anterior observations it would be reasonable to connect the activities of the Golgi body with the elaboration of the mucus. At any rate it is safe to say that there is no " degeneration " of the protoplasm, as the mucus-producing cells present features exhibited by well-known glandular secreting cells, viz. formation of droplets in a localized area above the nucleus, with migration of them towards the luminal border of the cell, and increase of Golgi material during this elaboration. Their final discharge appears to be of the same nature as that of the sebaceous glands, *i. e.* by cellular disintegration, with liberation of the contained contents, though it is probable that mucus is being discharged from the surface cells before final breakdown. Mitochondrial changes do not bear any obvious relationship to these cellular activities. The phenomena discussed are an interesting example of physiological metaplasia taking place under the influence of a hormone (? corpus luteum), the presence of which is capable of modifying the metabolic activities of the cells away from the production of eleidin to that of mucus. The chemistry of keratin compounds does not appear to be sufficiently known to make any useful correlation between them and the mucins possible, *i. e.* the alterations in synthetic ability of the cells cannot be evaluated in chemical terms.

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THE INFLUENCE OF NUTRITION ON SUSCEPTIBILITY TO A BACTERIAL TOXIN.

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Received for publication June 3rd, 1932.

THE object of this communication is to record certain experimental observations which illustrate the influence of nutritional factors on the susceptibility of animals to a bacterial toxin.

These observations were made in the course of a large-scale experiment on sheep living under natural environmental conditions at Garrochoran, an Argyllshire hill farm. This work has already been alluded to in a previous paper by Orr, Macleod and Mackie (1931), and its economic aspects are more fully described by Orr and Fraser (1932). During the first year of preliminary observation it was found that the nutritional value of the pasture was very low as judged by the production of the sheep stock it carried. Chemical analyses of the grass showed it to be deficient in several respects when compared with cultivated pasture, as indicated by the following data :

	Figures expressed as percentages of dry matter.			
	CaO.	P_2O_5	Cl.	N.
Cultivated pasture .	1·00	0·74	0·95	2·83
Garrochoran pasture	0·42	0·51	0·78	2·74

The figures for cultivated pasture are those quoted by Orr (1929) ; those for the Garrochoran pasture are the data obtained from samples collected in August, 1930, and analysed by Godden at the Rowett Institute. It is evident that the Garrochoran pasture is low in lime, phosphorus and chlorine when compared with cultivated pasture, and that this deficiency is particularly noteworthy in respect of lime. In addition to these chemical evidences of mineral deficiency, it was also noticed that there was a great scarcity of grass of any kind in late winter and early spring.

It was thought possible that the low productivity and high death-rate of the sheep stock might be due (among other factors) to the low mineral content of the herbage and to the quantitative scarcity of winter grass.

In order to investigate these possibilities, the hill was fenced off into four separate portions, and the sheep thus divided into four separate groups. In one group a ration of maize was given during the winter to test the effect of raising the caloric value of the diet. In a second group a mineral supplement containing lime, phosphorus and chlorine was given in sufficient quantity to bring the digestible mineral content of a day's ration of Garrochoran pasture up to that of a day's ration of cultivated pasture. The quantities necessary were calculated on the assumption that a sheep consumes 2 lb. of dry

matter daily, and on the digestibility figures available for the commercial mineral feeding stuffs. Cod-liver oil was added to increase the utilization of the lime and phosphorus, and a small amount of bran and maize to render the mineral ration palatable. In a third group the rations of Groups 1 and 2 were combined. In the fourth (control) group no supplement was given. This control group had been found to yield the best production results in the previous pre-experimental year. The details of the various rations were as follows :

	Maize.	Bran.	Mineral mixture.	Cod-liver oil.
Group I	½ lb.	*Nil*	*Nil*	*Nil*
Group II	½ oz.	½ oz.	1½ oz.	½ c.c.
Group III	½ lb.	½ oz.	1½ oz.	½ c.c.

It must be emphasized that these rations were not designed to elucidate the effect of individual mineral elements or of vitamins. They were intended primarily in the case of Group I to correct a quantitative deficiency, in Group II a qualitative deficiency, and in Group III to correct both.

While the experiment was not such as to allow of a controlled analysis of the effects of individual nutritional factors, the different rations produced a marked differential effect on the mortality and growth of the sheep. This is made clear by the following data :

	Group I.	Group II.	Group III.	Group IV.
Number of ewes	89	156	138	73
Percentage mortality of ewes	11	11	11	24
,, birth-rate of lambs	83	79	82	67
,, mortality of lambs	20	11	18	26
Average birth-weight of lambs in lb.	6	6	7	6
,, weight of lambs at weaning	50	49	52	48
,, fleece-weight of ewes in lb.	3·7	3·8	4·0	3·1

It will be seen that in every respect Group III (complete supplementary ration) is definitely superior to Group IV (pasture only), Groups I and II being intermediate.

Each individual sheep was weighed at intervals of a month throughout the year, except in April, when lambing was in progress. The different rations produced different growth-rates. During the winter all groups lost weight. Group III the least, Group IV the most, Groups I and II being intermediate. In summer all groups regained weight, but retained the same order : III>[I and II]>IV. Moreover, in condition, strength, activity and general health the groups showed a similar differentiation corresponding to the more easily measurable characters, such as weight and mortality.

The question arose whether the four groups might also show differences in their susceptibility to infective disease. Both for reasons of economy and the continuation of the main experiment, it was deemed inadvisable to attempt to ascertain this by any method involving experimental infection. It was therefore decided to test by cutaneous reactions their susceptibility to a bacterial toxin, and for this purpose the toxin of the lamb dysentery bacillus (Dalling, 1928) was selected. This organism, allied to *B. welchii*, forms in

culture an active exotoxin known to produce distinct reactions on intra-cutaneous injection. Reference has been made recently by Glenny, Llewellyn-Jones and Mason (1931) to the cutaneous reactions produced in laboratory animals by the toxin of *B. welchii* and related organisms, and they quote earlier work on the subject.

METHODS.

A dried preparation of the toxin was obtained by the courtesy of the Director of the Wellcome Laboratories, and preliminary tests showed that 0·5 c.c. of a 1 in 1000 dilution, when injected intra-cutaneously in sheep, produced a marked reaction consisting of a swollen purplish area associated with a varying amount of surrounding œdema. To gauge susceptibility varying dilutions of the toxin were used—1 in 50,000, 1 in 10,000 and 1 in 1000. The injections were made in the skin of the tail. The results were observed after 24 and 48 hours.

RESULTS.

A preliminary test was carried out with three ewe hoggs (1 year old) from each group, and striking differences were elicited, as shown in Table I. The animals of Group IV exhibited an unusually high susceptibility to the toxin as compared with the other groups.

TABLE I.—*Cutaneous Reactions of Sheep to the Toxin of the Lamb Dysentery Bacillus; Ewe Hoggs (1 year); May 30th, 1931. Results after 24 Hours.*

Group.	Number of sheep.	Dilutions of toxin injected. 1 : 1000.	1 : 10,000.	1 : 50,000.
I	4	+++	++	+
	60	+++	++	−
	27	++	+	−
II	108	+++	+	−
	215	+++	−	−
	229	+++	+	+
III	321	+++	−	−
	334	+++	+	−
	384	+++	+	−
IV	419	++++ with spreading œdema	+++	++*
	408	+++	+++	+
	433	++++ with spreading œdema	+++	++

* Died after 36 hours with general toxic symptoms.

The degree of intensity of the reaction is indicated by the number of + marks; any doubtful reactions have been classified as negative.

On June 6th a further series of tests were made in which 18–20 animals from each group were tested as before, though to avoid possible fatalities only a certain number were given the 1 in 1000 dilution. The results are summarized in Table II. The differentiation between Groups III and IV was well marked. As judged by the number of animals reacting to the 1 in 50,000

dilution of toxin the susceptibility was in the following serial order : IV>I>II >III.

TABLE II.—*Cutaneous Reactions of Sheep to the Toxin of the Lamb Dysentery Bacillus ; Sheep of Varying Age ; June 6th ; Results after 24 Hours.*

Group.	Numbers tested.	Numbers giving positive reactions to toxin diluted.		Tested also with 1 : 1000 dilution.
		1 : 10,000.	1 : 50,000.	
I	18	17	3	10—all reacted.
II	20	20	1	9—,, ,,
III	18	14	0	7—,, ,,
IV	20	20	9	9—,, ,, and one developed general toxic symptoms and died.

These sheep varied in age and in order to render the groups as comparable as possible in the ages of the animals tested, particularly as regards the younger sheep, another series were tested on June 27th. These consisted of 1- and 2-year-old animals (hoggs and gimmers). By this time, however, all animals, including Group IV, were obtaining an abundant diet of young grass, which was growing freely. A considerable proportion of the new series of animals had also been " wintered away " from the hill farm. The results are summarized in Table III, and it will be noted that there was little or no difference in susceptibility among the groups. It is possible that the change in the pasture

TABLE III.—*Cutaneous Reactions of Sheep to Toxin of Lamb Dysentery Bacillus ; Sheep of One and Two Years ; June 27th. Results after 24 Hours.*

Group.	Numbers tested.	Numbers giving positive reactions to toxin diluted.	
		1 : 10,000	1 : 50,000.
I	12	11	3
II	9	8	2
III	13	13	1
IV	9	9	2

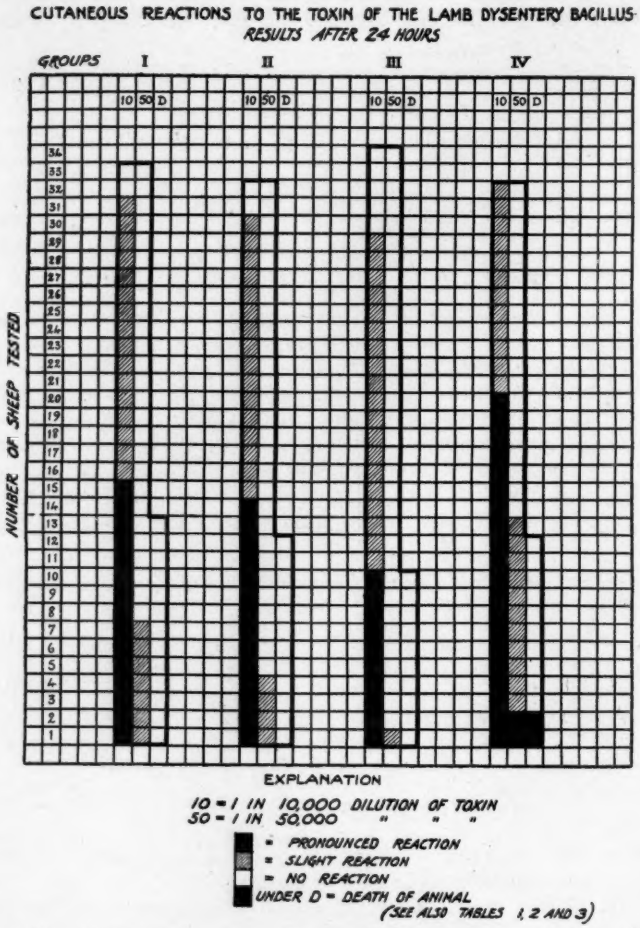
during the month of June accounted for the lack of differentiation which contrasted with that observed in the earlier tests.

The chart shows graphically the comparative results of the total tests carried out, including those on June 27th. Though these last have been included and tend to render the differentiation less striking, it is, nevertheless, well marked.

The significance of these differences was tested by the (P_χ^2) method, in which P is the probability that an equal or greater difference than that observed would be likely to occur in a population which was admittedly homogeneous. If, for example, $P = 0{\cdot}01$, a difference as great as or greater than the observed is likely to occur once in every 100 sets of observations. It is usually safe to draw a conventional line at $P = 0{\cdot}05$, and to consider that

any lesser value indicates a real discrepancy (see Pearson, 1914, and Yule, 1922). The order of susceptibility was IV>I>II>III.

There was no apparent relationship between susceptibility and age.



Fifteen sheep from each group all one and a half years old were tested also in October, 1931, but, as on June 27th, no significant difference could be elicited between the groups.

DISCUSSION AND SUMMARY.

The observations recorded in this communication, though limited in extent, appear to be of some significance in regard to the question of the influence of dietary factors on resistance to microbic infection.

Four different rations given to separate groups of sheep produced a clear differentiation in growth, in death-rate, and in general health. Judged by these characteristics, the four groups took the following order :

Group III (hill pasture + maize + mineral mixture)	Best.
Group I (hill pasture + maize)	Intermediate.
Group II (hill pasture + mineral mixture)	
Group IV (hill pasture only)	Worst.

Their resistance to the toxin of the lamb dysentery bacillus (judged by cutaneous reactions) was in the same order. This group variation was clearly marked in the early summer, less distinct in late summer, and disappeared in autumn. The seasonal change was in all probability related to the nutritive superiority of summer pasture.

This communication affords evidence that dietary deficiencies increase susceptibility to the action of a bacterial exotoxin. It does not offer any real evidence as to the relative importance of individual dietary factors in this respect. The observations recorded are only of a preliminary nature, but they suggest a profitable line of study in regard to the problem of the relation of nutritional factors to immunity in infective disease.

Acknowledgments.—We are indebted to Lt.-Col. McKendrick, Superintendent of the Laboratory of the Royal College of Physicians, for his guidance in checking the significance of our data by statistical methods ; also to Dr. O'Brien and Mr. Dalling, of the Wellcome Laboratories, for the preparation of toxin used.

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FURTHER OBSERVATIONS ON BACTERIAL CATAPHORESIS.

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Received for publication July 11th, 1932.

IN the considerable amount of literature upon the subject of bacterial cataphoresis there appears to be only scanty reference to the fact that certain strains of any one species differ very markedly in their behaviour from one another. One is somewhat inclined to gather from the literature that if under certain circumstances a particular strain of *B. typhosus* migrates at a definite rate in an electric field any other strain will, in all probability, behave in a similar manner. Mudd, Nugent and Bullock (1932) in their exhaustive and critical review of our modern knowledge of the physical chemistry of bacterial agglutination and its relation to colloidal theory refer to having worked with three strains of bacteria with negligibly small intrinsic surface charges.

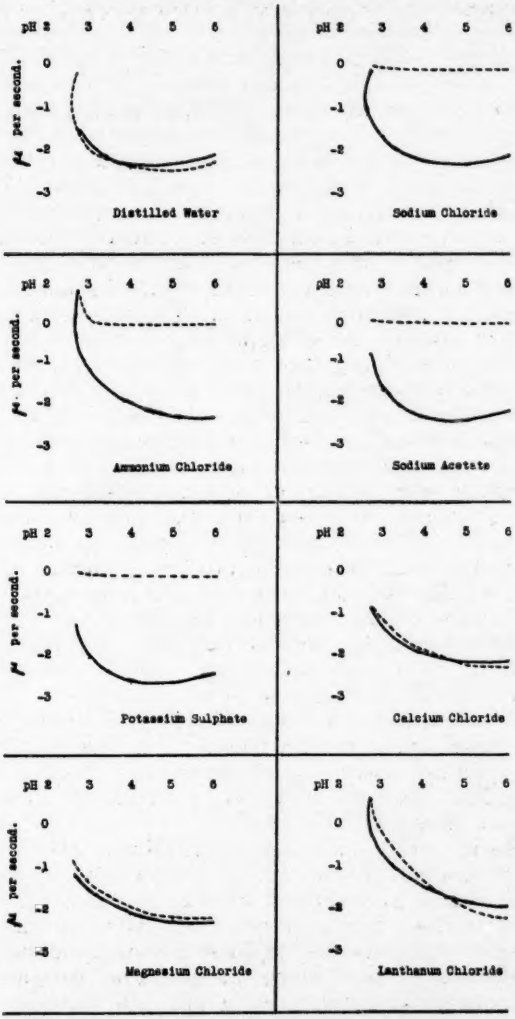
In the course of the last few years we have investigated a large number of strains of the typhoid-salmonella group, and have found very great variations in their cataphoretic behaviour. This variation may show itself in the following ways :

(1) All the individual bacteria migrate at a uniform speed.
(2) There is great variation among the migration rates of individuals.
(3) The bacteria remain practically stationary.

In the second and third instances it is quite impossible to record the migration rates.

With regard to the great irregularity in the migration rates of the bacteria in some cultures, it might be assumed that this was due to an admixture of bacteria having a high P.D. with others in which the charge was less ; if, however, such a culture is plated out and observations are again made upon cultures derived from single colonies, it will be seen that these too show the same type or irregularity as is present in the parent culture. Similarly, in cultures which are almost stationary when exposed to comparatively high voltages, we have not been able, by plating out and taking cultures from 24 single colonies, to get any cultures which behave differently from the parent culture. The most interesting deviation from the normally reacting culture is that type of culture which, when washed and suspended in distilled water, and subjected even to a pressure of 200 volts or more, shows practically no movement. At first it appeared likely that the technique was at fault and that there was some obstruction to the current, but we had no difficulty in showing that once a culture behaved in this way it would invariably continue to do so. Repeated subculture as well as culture on various media have no effect on the apparently relatively low negative charge of the organism. Only on one occasion have we observed the change in a culture from one which migrated normally to the form which became more sluggish and, later, after several more subcultures,

The effect of heating 24 hour cultures of Eberthella Basket in equinormal solutions of various salts.



The dotted line = the heated suspension

The continuous line = the unheated control

became almost stationary, even when subjected to high voltages. Apart from this instance it has been our experience that individual organisms in cultures of the typhoid-salmonella group will invariably either migrate uniformally,

show gross irregularity in this respect, or will almost fail to migrate even when exposed to high voltages So far we have failed in any attempt by exposure to solvents, such as alcohol or acetone or acids or alkalies, in transforming one type into the other We have failed to demonstrate any correlation between these different types of bacteria and their stability in suspension, neither have we been able to show that there is any material difference in the degree of hydration of the strains of the typhoid-salmonella group which we have investigated, as evidenced by the stability of suspensions of these organisms when acted upon by varying concentrations of tannin.

During the investigation of the possible causes of the variations in migration rates we found that suspensions of organisms in normal saline with a highly negative charge became almost stationary when these suspensions were heated to 56° C. When, however, a suspension of the same organism was made in distilled water and heated for the same time, viz., 2 hours at 56° C., no alteration in the migration rate was observed. The minimum concentration of sodium chloride to produce any effect was N/500,000. A culture which had been heated in this at 56° C. became very irregular and when the concentration was increased to N/100,000 the suspension became practically stationary. The time necessary for this change to take place appeared to be five minutes. The effect of heating bacteria in solutions of other salts was then investigated, and we found that the result was directly correlated with the valency of the cation. Bacteria heated in presence of sodium chloride, sodium nitrate, sodium sulphate, sodium acetate, potassium chloride, potassium nitrate, ammonium chloride and lithium sulphate, failed, when re-suspended in acetic acid-acetate buffers, to show any appreciable movement, even with as much pressure as 228 volts, corresponding to a voltage drop of 11·2 volts per centimetre. Heating in equimolecular concentrations of salts containing polyvalent cations produced no apparent effect on the migration rate of the bacteria as compared with that of the unheated controls. These results are shown in the accompanying figure.

The effect of heating a bacterial suspension in a 0·9 per cent. sodium chloride solution at varying temperatures for half an hour, was as follows : At 40° C. no alteration in the migration rate was observed ; at 45° C. a considerable amount of irregularity was produced ; at 50° C. the majority of the bacteria showed only slight movement.

From the above, and from the fact that heating at 56° C. for 5 minutes in solutions of salts with monovalent cations is necessary to reduce the charge of a bacterial suspension, it would appear that the time and temperature required is similar to that which produces death of the organism. It, therefore, appeared of interest to test the effect of killing the organism by other means than by heat in presence of salts of mono- and di-valent cations. The result in this case was similar to that in which heat was used, for when a suspension of *B. typhosus* was treated for 30 minutes at room temperature in a 1 per cent. solution of phenol in 0·9 per cent. NaCl a considerable number of the bacteria remained stationary and the remainder showed considerable irregularity in their migration rates. If, however, an equimolecular solution of calcium chloride was used in combination with the same concentration of phenol, no such alteration in the charge was produced.

SUMMARY.

1. The occurrence and persistence of certain irregularities in the migration rates of the individual members of typhoid-salmonella group has been described.

2. Numerous strains have been observed which fail to migrate even in a strong electric field.

3. The heating of a suspension of bacteria having a high negative charge in solutions of salts with monovalent cations reduces this charge approximately to zero, whereas no such effect is obtained if the cation is polyvalent.

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MIGRATION RATE AND VIRULENCE OF *C. DIPHTHERIÆ*.

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Received for publication June 28th, 1932.

JENSEN and Falk (1928) put forward a theory to explain the variations in toxigenicity which occur among strains of *C. diphtheriæ*. They assumed that all strains have equal powers of elaborating toxin but that only those whose surfaces are relatively permeable to toxin can excrete it into the medium. The more permeable strains would therefore be more toxigenic.

It was shown by Donnan (1911) that if two solutions are separated by a membrane which is impermeable to one of the ions present though freely permeable to all the others, a state of equilibrium is reached in which there is a difference in concentration, inside and outside the membrane, of the ions to which it is permeable. This difference in concentration of ions gives rise to a potential difference between the solutions on opposite sides of the membrane, which can be calculated and measured by appropriate methods.

In the opinion of Falk and his co-workers, the semi-permeability of the bacterial surface which their theory postulates should cause the establishment of a Donnan equilibrium, and hence a potential difference between the contents of the bacterial cell and the suspending medium. The potential difference should be least when the membrane is most permeable, *i. e.* for the toxigenic strains. They considered that evidence bearing on this point could be obtained by correlating the virulence of a number of strains as found by animal inoculation with the rates at which they migrate in an electric field, since the electrophoretic mobility is assumed to be dependent on, and hence a measure of, the potential difference at the surface of the bacillus.

There appears, however, to be a fallacy in this deduction : the potential difference resulting from a membrane equilibrium—such as is considered in

Falk's theory—is the thermodynamic potential transversely across the membrane, while that measured by cataphoresis is the electro-kinetic potential and is tangential to the surface. Far from being identical Kruyt (1930) states that these two potentials may be opposite in sign and may vary independently as conditions are altered.

The results obtained by Falk and his co-workers cannot therefore be taken as bearing on the theory they propound. They state, however, " it is clearly recognized that although the investigations described here were planned on the hypothesis outlined, the validity of the experimental findings is in no wise dependent upon the validity of the theoretical treatment. Statements made here to describe a working hypothesis are not intended to be a commitment to the theory that P.D. or charge necessarily has origin in a membrane potential."

A number of experiments performed by these observers appeared to show that non-virulent corynebacteria always migrated more rapidly than virulent strains. Falk therefore suggested a diagnostic method in which the migration rates of suspected strains were compared with the rates of known toxigenic, feebly toxigenic and non-toxigenic cultures tested at the same time and under the same conditions. These three types should give a range of ascending velocities and the diagnosis of an unknown strain depended on the position its migration rate occupied with reference to these three standards.

When the method was used for a large series of recently isolated strains (Falk, Jensen and Mills, 1928) agreement with animal tests was obtained in 94·4 per cent. of the cultures : in only one instances was a strain, virulent by inoculation, missed by cataphoresis ; in the other cases of disagreement the electrophoresis test erred on the side of safety by considering virulent, strains which were avirulent by animal experiment.

This work has been repeated by other observers with varying results : Stone and Weigel (1929) tested a series of 87 strains by cataphoresis and by animal inoculation and found that the highest migration rates belonged to virulent strains, and the lowest to non-virulent cultures, while between the extremes the two types were intermingled.

Thompson (1931), who used phosphate buffer instead of distilled water as the suspending medium, agreed with Falk's main contention that virulence varies inversely with migration rate, but concluded that since there is no sharp line of demarcation between the two types and the experimental error is relatively high, the method could not be applied to routine procedure.

Jones (1931) examined 34 cultures of *C. diphtheriæ* by both methods and found no correlation at all between migration rate and toxigenicity. He also isolated several aberrant avirulent types from virulent cultures but these showed no significant change in P.D.

Since any means by which virulent and non-virulent strains could be differentiated without animal inoculation would be of the greatest practical value, Dr. H. J. Parish, of the Wellcome Physiological Research Laboratories, offered to co-operate with us in an attempt to reproduce the findings of Falk and his co-workers, and supplied us with all the strains which we have examined. The cultures, chiefly recently isolated strains, were known to us only by numbers until all our tests had been completed after which the results of virulence tests

as found by Dr. Parish were communicated to us for comparison : " virulent " means " lethal on subcutaneous injection," or " producing necrosis on intradermal injection " into guinea-pigs (Okell and Parish, 1926).

Method.—The bacillary growth from 24-hour Loeffler slopes or 48-hour veal broth cultures was washed three times and suspended in distilled water or M/150 phosphate buffer of pH 7·0. Using the type of cataphoresis cell described by us (Brown and Broom, 1929) the migration rates of 10 individual bacteria in the stationary layer were examined. The cell was washed out, refilled from the same stock suspension and a second set of observations made. This procedure was repeated again giving in all 30 observations in groups of 10. This method was adopted because it was found to provide a useful check on the accuracy of working in that a variation of more than a few tenths of a second among the means of these three sets of observations could always be traced to some error in technique.

Table I shows the degree of accuracy we were able to obtain when cultures were examined at an interval of a few days.

TABLE I.

	Date.	Migration rates in μ per second per volt per centimetre. Mean of 10 observations.		
		1st reading.	2nd reading.	3rd reading.
Culture A	30.xi.31	2·95	2·95	2·91
	3.xii.31	2·97	2·97	3·00
Culture B	30.xi.31	3·13	3·16	3·19
	31.xii.31	3·13	3·13	3·13

Before testing a large series of diphtheria strains, 4 avirulent and 7 virulent cultures were examined under different conditions of growth and suspending media as shown in Table II, the results being expressed as velocities of migration.

TABLE II.

Culture.	Virulence.	Migration rates in μ per second per volt per centimetre.			
		A.	B.	C.	D.
C.D. 120	V.	1·88	2·94	1·72	1·75
,, 121	V.	1·50	2·22	1·98	1·66
,, 123	V.	1·49	2·31	1·72	1·79
,, 124	V.	1·62	2·58	1·75	1·83
,, 4253	AV.	2·02	3·54	1·76	1·81
,, 4549	AV.	2·16	3·38	1·58	1·95
,, 4569	AV.	1·62	3·12	1·72	1·55
,, 4580	V.	1·72	2·56	1·52	1·73
,, 4586	AV.	1·96	2·51	1·68	1·63
,, 4591	V.	1·80	2·51	1·65	1·75
,, 4593	V.	2·06	2·32	1·96	1·86

A. = Suspension in distilled water of 24-hour Lœffler slope culture. B. = Suspension in M/150 phosphate buffer of 24-hour Lœffler slope culture. C. = Suspension in distilled water of 24-hour veal broth culture. D. = Suspension in distilled water of 48-hour veal broth culture. V. = Virulent. AV. = Avirulent.

It appeared from this experiment that a distinction between the two types was most likely to be found by studying the migration rates of cultures grown for 24 hours on Loeffler slopes and suspended in M/150 phosphate buffer of pH 7·0. Falk, however, states that 48-hour veal broth cultures should be used and we have therefore tested a number of our strains under both sets of conditions. Tables III and IV show respectively the migration rates of the strains, tested in distilled water and in M/150 phosphate buffer of pH 7·0, compared with the results of the virulence tests as obtained by Dr. Parish.

TABLE III.—*Virulence Compared with Migration Rate in Distilled Water of 38 Strains from 48-hour Veal Broth Cultures.*

Culture.		Migration rate in aq. dest.	Culture.		Migration rate in aq. dest.
X. 3	(AV.)	3·08	C.D. 231	(V.)	2·16
C.D. 4698	(V.)	2·90	,, 119	(V.)	2·14
X. 1	(AV.)	2·74	,, 4210	(AV.)	2·10
X. 2	(AV.)	2·74	,, 240	(V.)	2·08
C.D. 198	(V.)	2·71	,, 103	(V.)	1·98
,, 230	(V.)	2·68	,, 4549	(AV.)	1·95
,, 193	(V.)	2·63	,, 241	(AV.)	1·90
,, 229	(V.)	2·61	,, 109	(V.)	1·88
,, 3378	(AV.)	2·56	,, 4593	(V.)	1·86
,, 206	(V.)	2·54	,, 124	(V.)	1·83
,, 4221	(AV.)	2·50	,, 4253	(AV.)	1·81
,, 242	(V.)	2·48	,, 123	(V.)	1·79
,, 4766	(V.)	2·42	,, 4591	(V.)	1·75
,, 243	(V.)	2·40	,, 120	(V.)	1·75
,, 4227	(AV.)	2·40	,, 4580	(V.)	1·73
,, 232	(V.)	2 37	,, 106	(V.)	1·71
H. 3	(AV.)	2·36	,, 121	(V.)	1·66
C.D. 189	(V.)	2·21	,, 4586	(AV.)	1·63
,, 110	(V.)	2·18	,, 4569	(AV.)	1·55

Under the conditions of these tests there is no evidence of any correlation between virulence and migration rate.

Dr. R. A. O'Brien suggested that it might be of interest to distinguish between "gravis" and "mitis" strains (Anderson, Happold, McLeod and Thomson, 1931) because it has been the experience at the Wellcome Physiological Research Laboratories that "gravis" are poorly toxigenic, while "mitis" strains have a capacity for much greater toxin production under the conditions in which diphtheria toxin is usually produced in laboratories: the "gravis" strains therefore should migrate more rapidly than the "mitis" if migration rate varies inversely with toxigenicity. No such correlation was found, the "gravis" and "mitis" types being distributed apparently at random throughout the range of velocities.

Although we were able to repeat our findings with a considerable degree of accuracy after a short time interval it was found that, after from 6 weeks

TABLE IV.—*Virulence Compared with Migration Rate in M/150 Phosphate Buffer pH 7·0 from 24-hour Loeffler Slopes.*

Culture.		Migration rate in buffer.	Culture.		Migration rate in buffer.
C.D. 232	(V.)	3·51	C.D. 243	(V.)	2·46
,, 4406	(AV.)	3·46	,, 4227	(AV.)	2·45
,, 110	(V.)	3·24	,, 200	(V.)	2·42
,, 268	(V.)	3·17	,, 241	(AV.)	2·40
,, 266	(V.)	3·14	,, 240	(V.)	2·40
,, 119	(V.)	3·12	,, 4220	(AV.)	2·40
,, 4549	(AV.)	3·04	,, 252	(V.)	2·38
H. 1	(AV.)	2·92	,, 270	(V.)	2·34
C.D. 4759	(AV.)	2·87	,, 4593	(V.)	2·32
,, 4210	(AV.)	2·86	,, 251	(V.)	2·32
,, 265	(V.)	2·84	,, 123	(V.)	2·31
,, 4226	(AV.)	2·84	,, 245	(V.)	2·29
,, 248	(V.)	2·78	,, 253	(V.)	2·28
,, 4569	(AV.)	2·78	,, 250	(V.)	2·28
,, 120	(V.)	2·72	,, 254	(V.)	2·27
,, 276	(V.)	2·71	,, 230	(V.)	2·25
,, 4698	(V.)	2·71	,, 229	(V.)	2·23
H. 3	(AV.)	2·71	,, 231	(V.)	2·23
X. 3	(AV.)	2·68	,, 206	(V.)	2·23
C.D. 255	(V.)	2·64	,, 272	(V.)	2·23
,, 264	(V.)	2·64	,, 121	(V.)	2·22
,, 275	(V.)	2·64	,, 249	(V.)	2·16
,, 4253	(AV.)	2·63	,, 4600	(AV.)	2·07
X. 1	(AV.)	2·62	,, 274	(V.)	2·07
C.D. 124	(V.)	2·58	,, 193	(V.)	2·05
,, 4766	(V.)	2·56	,, 109	(V.)	1·98
,, 4580	(V.)	2·56	,, 278	(V.)	1·98
,, 4774	(V.)	2·56	,, 106	(V.)	1·92
X. 2	(AV.)	2·54	,, 103	(V.)	1·92
C.D. 4586	(AV.)	2·51	,, 198	(V.)	1·89
,, 4591	(V.)	2·51	,, 4221	(AV.)	1·88
,, 242	(V.)	2·50	,, 269	(V.)	1·86
,, 4781	(AV.)	2·47	,, 244	(V.)	1·84
,, 246	(V.)	2·46	,, 189	(V.)	1·40
,, 3378	(AV.)	2·46			

to 2 months, some of the strains in this series showed definite variations from their original velocities.

Both the virulent and the avirulent types have been affected, but we are not at present able to state whether this change is accompanied by alterations in other characteristics: the occurrence of such a variation, however, seems to increase the difficulties of basing a routine diagnostic procedure on a cataphoresis test.

SUMMARY.

1. The question whether cataphoresis studies will afford evidence bearing on the theory propounded by Falk and his co-workers to explain variations in toxin production by different strains of diphtheria bacilli, is discussed.

2. The migration rates of 69 strains of corynebacteria were examined under different conditions.

3. No evidence was obtained for any correlation between these rates and the virulence of the same strains tested by an independent observer.

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THE ESTIMATION OF SMALL AMOUNTS OF A SPECIFIC BACTERIAL POLYSACCHARIDE BY THE INDUCTION OF ANAPHYLAXIS.

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Received for publication July 11th, 1932.

THE production of bacterial anaphylaxis was clearly demonstrated by Rosenau and Anderson as long ago as the year 1907, and has been studied by many workers since that time. Weil and Torrey (1916) showed that guinea-pigs when inoculated with the serum of patients who were suffering from pneumonia frequently became hypersensitive to autolytic extracts of pneumococci. The sensitizing action of the serum was evident when it was collected from the patients during the febrile stage of the disease; the serum was active in only 2 out of 20 cases examined when it was collected during convalescence. Zinsser and Mallory (1924) found that guinea-pigs which were actively sensitized with massive doses of heat-killed pneumococci became susceptible to anaphylaxis when alkaline extracts of the homologous organism were injected later. In the experiments just cited sensitization was recognized by the reaction of the guinea-pig's uterus when it was submitted to the Schultz-Dale test. Mackenzie (1924) showed that guinea-pigs which had been actively

immunized with killed and living cultures of Type I pneumococcus died with typical anaphylactic shock when a small injection of the homologous non-toxic culture filtrate was given intravenously. In all these experiments autolytic extracts or alkaline solutions of the bacterial cells were used, and it was not possible to state whether the specific or non-specific polysaccharide or protein substances had produced the anaphylactic shock.

The first attempt to bring about anaphylaxis with purified bacterial polysaccharides was made by Tomcsik (1927), who used the specific carbohydrate substance he obtained from *B. lactis aërogenes*. The injection of 0·033 mgm. of this substance, which contained 0·9 per cent. of nitrogen, caused fatal anaphylactic shock in guinea-pigs which had been passively sensitized with the homologous antibacterial serum. Later Tomcsik and Kurotchkin (1928) achieved similar results by using specific polysaccharides which they isolated from the pneumobacillus of Friedländer and from yeast-cells. Lancefield (1928) found that the species-specific "C" substance which she isolated from various strains of hæmolytic streptococci and which was probably carbohydrate in nature, gave rise to anaphylaxis in passively sensitized guinea-pigs. The polysaccharide preparations used in all these experiments contained nitrogen, and the authors realized the possibility that the anaphylactic shock they observed might have been due to traces of protein which they had not succeeded in eliminating during the process of purification. The experiments of Avery and Tillett (1928), who used the highly purified bacterial polysaccharides isolated by Heidelberger, Goebel and Avery (1925) from pneumococcus Types II and III, were not open to this criticism. Avery and Tillett furnished clear proof that the nitrogen-free bacterial polysaccharides of the pneumococcal types with which they worked produced typical and fatal anaphylactic shock in guinea-pigs when these were passively sensitized with the homologous precipitating rabbit-serum.

The present paper records anaphylaxis experiments in guinea-pigs with the purified specific polysaccharide isolated from *B. dysenteriæ* (Shiga) (Morgan, 1931); the animals were passively sensitized with the homologous antibacterial serum. Experiments are also described in which the production of anaphylaxis in sensitized guinea-pigs has been used as a test to identify the presence of the specific polysaccharide in various polysaccharide fractions which have been obtained during the isolation of the purified specific substance.

The following data summarize the chemical and immunological properties of the specific and the non-specific bacterial polysaccharides which have been used in this work. A preliminary account of the method of isolation of the specific polysaccharide has already been given.

The specific polysaccharide is weakly acidic, and is strongly dextro-rotatory $[\alpha]^{20^\circ}_{5461} + 107^\circ$. It contains approximately 1·8 per cent. nitrogen, which is, however, not liberated by the prolonged action of nitrous acid in the presence of dilute mineral acid. The specific substance is not precipitated from aqueous solution by normal lead acetate, barium hydroxide, phospho-tungstic acid, silver nitrate or Fehling's solution; it gives specific precipitation with the homologous immune serum up to a dilution of 1 in 12,000,000. Hydrolysis of the specific polysaccharide with acid gives rise to 100 per cent. of reducing

substances, as measured by the sodium hypoiodite method of Macleod and Robison (1929). From the hydrolysis products galactose and an acetylaminohexose have been isolated.

All attempts to eliminate the nitrogen from the polysaccharide-complex without destroying the specific properties have failed. The nitrogen-content cannot be due to combined protein, since 1·8 per cent. of nitrogen corresponds to between 10 per cent. and 15 per cent. of admixed protein in the polysaccharide preparation, whereas the material has failed to yield any positive test for proteins, even when 40 mgm. amounts of the substance were used in the tests. Furthermore, the specific polysaccharide regenerated from the acetyl or the benzoyl derivative, or the material recovered after the prolonged action of nitrous acid, still contains about 1·8 per cent. of nitrogen. It seems, therefore, that the preparations of the specific polysaccharide used in the experiments described in this paper were free from protein.

The non-specific bacterial polysaccharide $[\alpha]_{5461}^{20^\circ} - 47^\circ$ is nitrogen-free and is completely precipitated by phospho-tungstic acid. The absence of this polysaccharide from the culture medium before it has been inoculated with culture makes it appear probable that this substance is a true somatic constituent of Shiga's bacillus or that it is a product of its metabolism. This polysaccharide gives no precipitation at any dilution with antibacterial rabbit sera prepared against killed cultures of either the " rough " or the " smooth " variant of *B. dysenteriæ* (Shiga).

EXPERIMENTAL.

The Determination of the Minimal Anaphylactic Dose of the Specific Polysaccharide.

Guinea-pigs weighing 250 gm. to 300 gm. were passively sensitized by the intraperitoneal injection of 1 c.c., 2 c.c. or 3 c.c. of an antibacterial *B. dysenteriæ* (Shiga) rabbit-serum. The degree of sensitization was tested at an interval of 24, 38 or 72 hours after the intraperitoneal dose of serum by the intravenous inoculation of a solution of the specific polysaccharide in 0·9 per cent. saline. Table I shows the results of experiments which were carried out to determine the minimal anaphylactic dose (M.A.D.) of the specific substance (see Weil, 1913).

In all the cases examined the animals which died presented typical post-mortem appearances; the lungs were found to be bloodless and distended, and to fill the thorax completely.

It will be seen from the Table that the minimal anaphylactic dose is about 0·015 mgm. of the polysaccharide, and that this figure does not seem to vary whether the animal is passively sensitized with 1 c.c., 2 c.c. or 3 c.c. of the immune rabbit-serum. A dose of 0·010 mgm. of the specific substance allowed the survival of 3 out of 6 animals tested; this amount is, therefore, slightly less than 1 M.A.D. Ten mgm. of the polysaccharide is quite non-toxic when it is given intravenously to unsensitized guinea-pigs. The control animals, guinea-pigs 19 and 20 in the Table, which received a dose of normal rabbit-serum in place of immune serum, showed no symptoms of anaphylaxis when

TABLE I.—*Determination of the Minimal Anaphylactic Dose of the Specific Polysaccharide.*

Guinea-pig No.	Guinea-pig sensitized with intraperitoneal dose of: Serum.	Volume (c.c.).	Time-interval between passive sensitization and intravenous dose (hrs.).	Intravenous dose of specific polysaccharide (mgm.).	Result.
1	Immune	1·0	72	3·0	† (2½ minutes).
2	,,	,,	,,	0·500	† (3 ,,).
3	,,	,,	38	0·250	† (3½ ,,).
4	,,	,,	,,	0·100	† (2 ,,).
5	,,	,,	24	0·100	† (2½ ,,).
6	,,	,,	,,	0·025	† (3 ,,).
7	,,	,,	,,	0·020	† (3 ,,).
8	,,	,,	,,	0·020	† (2½ ,,).
9	,,	,,	,,	0·020	† (4 ,,).
10	,,	,,	,,	0·015	† (2½ ,,).
11	,,	,,	,,	0·015	† (3½ ,,).
12	,,	,,	,,	0·010	Severe shock, survived.
12*a*	,,	,,	,,	0·010	† (3 minutes).
13	,,	,,	,,	0·010	Shock, survived.
13*a*	,,	,,	,,	0·010	† (20 minutes).
14	,,	2·0	,,	0·020	† (4½ ,,).
15	,,	,,	,,	0·010	Shock, survived.
16	,,	3·0	,,	0·020	† (4½ minutes).
17	,,	,,	,,	0·010	† (35 ,,).
18	,,	,,	,,	0·010	Severe shock, survived.
19	Normal	1·0	,,	5·0	No shock.
20	,,	,,	,,	7·0	,,
21	"Rough"	,,	,,	2·0	,,
22	,,	,,	,,	0·10	,,

† Indicates death of animal.

5 mgm. or 7 mgm. (about 300 M.A.D.'s) of the specific polysaccharide was given intravenously. Guinea-pigs 21 and 22 were sensitized with an immune serum prepared against a whole killed culture of the corresponding "rough" variant of Shiga's bacillus. The complete absence of anaphylactic shock indicates that "rough" organisms of *B. dysenteriæ* (Shiga) contain very little, if any, of the "smooth" antigen.

Attempts to sensitize guinea-pigs with an antibacterial horse-serum possessing a high titre of precipitins against the purified specific polysaccharide of *B. dysenteriæ* (Shiga) have failed. The inability of horse-serum to sensitize guinea-pigs to a subsequent intravenous dose of polysaccharide substance was first observed by Avery and Tillett (1929) while investigating the induction of anaphylactic shock with the type-specific carbohydrates of pneumococcus (Types I, II and III).

From these experiments it appears that as little as 0·015 mgm. of the specific

substance of *B. dysenteriæ* (Shiga) can be detected by the production of a fatal anaphylactic shock in guinea-pigs which have been passively sensitized with an immune rabbit-serum.

The Detection and Quantitative Estimation of Small Amounts of the Specific Polysaccharide.

During the course of work on the isolation and purification of the specific and a non-specific polysaccharide from Shiga's bacillus other polysaccharide fractions were also obtained. The amounts of these fractions were small and it was not, therefore, possible to apply to them a chemical purification as thorough as that which had been used for the specific substance. Thus the extent to which these polysaccharide fractions were contaminated with the specific polysaccharide could not be determined by chemical means. The sharp and consistent results that were obtained in the experiments in which the minimal anaphylactic dose of several preparations of the specific Shiga polysaccharide were determined, suggested that an anaphylaxis test in guinea-pigs might prove to be useful for the detection and quantitative estimation of the specific substance in the presence of non-specific polysaccharide impurities.

TABLE II.—*Detection of the Specific Polysaccharide in the Presence of a Large Excess of Non-specific Carbohydrate Substance.*

Guinea-pig No.	Composition of mixture given intravenously.		Percentage of specific polysaccharide in mixture.	Result.
	Non-specific substance.	Specific polysaccharide.		
34	2·5 mgm. glycogen	0·10 mgm.	4·0	† (2½ minutes).
33	5·0 ,, ,,	0·05 ,,	1·0	† (3 ,,).
32	50·0 ,, ,,	0·05 ,,	0·1	Very severe shock, recovered.
35	10·0 ,, yeast gum	0·10 ,,	1·0	† (3 minutes).
35*a*	10·0 ,, ,,	0·02 ,,	0·2	† (3 ,,).
37	10·0 ,, gum arabic	0·10 ,,	1·0	† (4½ ,,).
37*a*	10·0 ,, ,,	0·02 ,,	0·2	† (4 ,,).
38	10·0 ,, lactose	0·10 ,,	1·0	† (4 ,,).
39	10·0 ,, starch	0·10 ,,	1·0	† (3 ,,).
39*a*	10·0 ,, ,,	0·02 ,,	0·2	† (3½ ,,).
40	10·0 ,, raffinose	0·10 ,,	1·0	† (3½ ,,).
40*a*	10·0 ,, ,,	0·02 ,,	0·2	† (4½ ,,).
41	10·0 ,, non-specific Shiga polysaccharide	0·10 ,,	1·0	† (2½ ,,).
41*a*	10·0 mgm. non-specific Shiga polysaccharide	0·02 ,,	0·2	† (3½ ,,).

† Indicates death of animal.

In order to ascertain whether a large excess of non-specific polysaccharide or carbohydrate substance might inhibit the lethal anaphylactic effect of

one or more minimal anaphylactic doses of the specific polysaccharide, tests were carried out with artificially prepared mixtures of the specific substance and various non-specific substances. It will be seen from Table II that in no instance was anaphylaxis inhibited, although in all but one test, guinea-pig 34, the specific substance represented not more than 1·0 per cent. of the carbohydrate mixture.

A non-specific polysaccharide, $[\alpha]_{5461}^{20^\circ} - 47^\circ$, isolated from *B. dysenteriæ* (Shiga), produces no inhibition of anaphylaxis when it is added to only one minimal anaphylactic dose of the specific polysaccharide (test animal 41*a*). This non-specific polysaccharide does not give rise to anaphylaxis even when 1·0 mgm. of it is given intravenously to guinea-pigs which have been passively sensitized by means of antibacterial sera prepared against either "rough" or "smooth" *B. dysenteriæ* (Shiga). Guinea-pig 32, which received 50 mgm. glycogen together with 0·02 mgm. of the specific polysaccharide was the only animal tested that survived a larger dose of the specific substance than 0·015 mgm.

TABLE III.—*The Quantitative Determination of Specific Polysaccharide in an Unknown Polysaccharide Mixture.*

Guinea-pig No.	Dose of mixture given intravenously. Volume c.c.	mgm.	Result.	Conclusion as to the percentage of specific polysaccharide contained in the mixture.
50	1·00	2·00	† (2 minutes)	More than 0·75
51	0·50	1·00	† (3½ ,,)	,, ,, 1·50
53	0·40	0·80	† (3½ ,,)	,, ,, 1·90
53*a*	0·40	0·80	† (4 ,,)	,, ,, 1·90
54	0·35	0·70	† (4 ,,)	,, ,, 2·10
54*a*	0·35	0·70	† (3 ,,)	,, ,, 2·10
54*b*	0·35	0·70	† (4½ ,,)	,, ,, 2·10
55	0·30	0·60	Severe shock, survived	Less than 2·50
55*a*	0·30	0·60	† (3 minutes)	,, ,, 2·50
55*b*	0·30	0·60	† (3½ ,,)	,, ,, 2·50
56	0·25	0·50	† (3½ ,,)	,, ,, 3·00
56*a*	0·25	0·50	Shock, survived	,, ,, 3·00
57	0·20	0·40	,, ,,	, ,, 3·70
57*a*	0·20	0·40	,, ,,	,, ,, 3·70
58	0·10	0·20	Very slight shock survived	,, ,, 7·50

† Indicates death of animal.

For the quantitative estimation of the specific polysaccharide in the presence of a large excess of non-specific polysaccharide substance, an artificial mixture of gum arabic and the specific substance was made. A solution of the mixture was prepared by dissolving 0·200 gm. of the dry material in 100 c.c. of 0·9 per cent. saline. The guinea-pigs were sensitized with 1 c.c. of immune

serum 24 hours before receiving intravenously decreasing volumes of the solution of the mixture. The results of the test are shown in Table III. The first four guinea-pigs tested received intravenously 1·0 c.c., 0·5 c.c., 0·4 c.c. and 0·35 c.c. of the solution respectively. These volumes correspond to 2·00 mgm., 1·00 mgm., 0·80 mgm. and 0·70 mgm. of the mixture. All the animals died with typical anaphylactic shock, a result which indicates that these amounts of the mixture contained at least one minimal anaphylactic dose, that is, at least 0·015 mgm. of the specific polysaccharide. Since, from this test, 0·70 mgm. of the mixture contained 0·015 mgm. of the specific substance, it follows that the mixture contained at least 2·1 per cent. of the specific substance. With smaller amounts of the mixture some of the test guinea-pigs survived after showing symptoms of anaphylactic shock. The largest dose of the polysaccharide mixture which, after producing very severe shock allowed the survival of some of the test animals, appeared to be 0·30 c.c., or 0·60 mgm. of the dry polysaccharide mixture. The survival of some of the guinea-pigs indicates that this amount of the mixture contained rather less than one minimal anaphylactic dose, that is, less than 0·015 mgm. of the specific substance. The tests as a whole indicate that the polysaccharide mixture contained more than 2·1 per cent. and less than 2·5 per cent. of the specific substance. The preparation actually contained 2·0 per cent. of the specific polysaccharide.

The accuracy of the final result depends on various factors; those of importance are, the percentage of specific substance in the mixture, the constancy with which the test animal reacts to a minimal anaphylactic dose of the specific substance, and the accuracy with which the minimal anaphylactic dose has been determined. In the example given it is possible to distinguish between a content of 2·1 per cent. and 2·5 per cent. of the specific substance, but it would probably be impossible to distinguish between 19·5 per cent. and 20·0 per cent. of specific substance in an unknown mixture.

The results described in this paper in conjunction with those of other workers already cited, suggest that the anaphylaxis test may be used for the quantitative estimation of small amounts of any specific bacterial polysaccharide. The method is specially suitable for the detection of a particular specific bacterial polysaccharide when examining polysaccharide fractions which are obtainable in only small amounts during the isolation of the pure specific substance. By the aid of this method it has been possible to ascertain whether particular polysaccharide substances which have been isolated from *B. dysenteriæ* (Shiga) contain 5 per cent., 10 per cent., or 15 per cent. of the specific substance, and the determinations have been made by the use of less than 10 mgm. of the mixed polysaccharide material.

The presence of a species-specific polysaccharide, such as has been recently described by Tillett, Goebel and Avery (1930) and Heidelberger and Kendall (1931) for the four pneumococcal types, is likely to complicate the anaphylaxis test when it is applied for the detection of the corresponding type-specific polysaccharide. It is reasonable, however, to suppose that a rigidly type-specific anaphylactic shock will result if the test animals are immunized with the corresponding "smooth" immune serum after absorption with the homologous "rough" organisms. Furthermore, the test should be applicable for the estimation of any species-specific polysaccharide, provided that animals

are used which have been passively sensitized with the homologous "rough" immune serum.

SUMMARY.

1. The purified specific polysaccharide which has been isolated from the "smooth" variant of *B. dysenteriæ* (Shiga) produces a typical and fatal anaphylactic shock in guinea-pigs which have been passively sensitized with the homologous antibacterial serum from the rabbit.

2. As little as 0·015 mgm. of the specific polysaccharide can be detected by the production of a fatal anaphylactic shock in the guinea-pig.

3. A method is described for the quantitative determination of the specific polysaccharide in the presence of a large excess of non-specific polysaccharide substance.

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EXPERIMENTAL INFLAMMATION OF THE COLON: (*a*) RELATIONSHIP OF MUCUS PRODUCTION IN GOBLET-CELLS TO THE GOLGI APPARATUS; (*b*) MITOCHONDRIAL CHANGES.

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Received for publication May 1st, 1932.

NASSONOV (1923) was apparently the first histologist to state clearly the view that the Golgi apparatus is intimately connected with the elaboration of secretion granules. Among other cells he showed that the mucin in the large goblet-cells of a Triton originated in connection with this body.

His observations were confirmed and extended by Bowen (1926) to other

* With a grant from the Medical Research Council.

cells. In a review by Parat (1928) the evidence is considered and criticized, the history of the idea being given. The reader is also referred to a review of the subject by McBride in 'Recent Advances in Cytology.' Of the more recent papers, that of Clara (1926) on the mucus-producing cells of birds is of considerable interest. He shows that in several species the mucin first appears in goblet-cells just above the nucleus. He does not emphasize from his observations the possibility of a connection with the Golgi apparatus. In the course of some work with Dr. Webb (Florey and Webb, 1931) certain observations suggested that there might be a topographical relationship between the Golgi apparatus and the first mucin granules to be produced in cells of the cat's colon.

This possibility has now been explored in several mammals, the results from five species being given here.

Histological technique.—In some of the earlier work "Susa" was used as a fixative, but it is now realized that this is unsuitable for obtaining good fixation of mucus. Three fixatives have been subsequently employed, after trials of many more: Altmann's fluid (equal parts of 5 per cent. potassium bichromate and 2 per cent. osmic acid); Nassonov's modification of Champy's fluid (2 parts of 3 per cent. potassium bichromate, 2 parts of 1 per cent. chromic acid and 1 part of 2 per cent. osmic acid); Regaud's fluid (20 c.c. formalin and 80 c.c. 3 per cent. potassium bichromate). Helly's fluid has also been used.

For purposes of mitochondrial fixation immersion fixation was found quite inadequate, even when the mucosa only of the cat's colon was used or the thin colon of a rat opened and fixed flat. Very good fixation of the cells, especially towards the bases of the crypts, was, however, obtained by intra-arterial injection of the fixative. In the case of the rat a cannula was fixed in the descending aorta of the animal just killed (by coal-gas). By means of a syringe warm physiological saline was gently perfused through the abdominal viscera and hind legs, exit for the fluid being made by a cut in the right auricle. When the fluid issuing was clear the syringe was filled with fixative, and this injected till the fixative was observed to be coming out of the venous exit; the latter was then clamped by a pair of artery forceps. The remainder of the fixative was then forced in very slowly and penetrated into the organs. In this way it was possible to get fixation with osmic acid—containing fixatives using as little as 8 c.c. The organs required were then excised and placed in more fixative and left 24 hours.

In the case of the rat the cannula was inserted into the inferior mesenteric artery, and a small length only of colon perfused after the requisite ligation of vessels. In this case 5 c.c. of injected fixative sufficed. The material in subsequent treatment was washed for 24 hours in running water and then passed rapidly through alcohols and xylol into wax, in which it remained a minimum time before casting.

As nuclear stains iron-hæmatoxylin of Heidenhain and hæmatoxylin of Carazzi have been employed. The latter is not very effective after osmic fixation, but sufficiently good pictures of the nucleus were obtained. Muci-carmine, made according to the directions in Masson's 'Diagnostiques du Laboratoire,' was employed as a specific stain for mucus, and, after all three

fixatives, gave excellent staining of the mucus. Muci-carmine after prolonged osmication, for the impregnation of the Golgi-bodies, loses its effectiveness. Metanil yellow was frequently used as a counterstain. Mitochondria were stained by Altmann's method, Benda's crystal violet, and iron-hæmatoxylin.

For the Golgi bodies both the osmic acid technique according to Nassonov and Cajal's silver impregnation method were employed.

All detailed observations were made with a Zeiss 1·5 mm. apochromatic lens with very critical illumination.

RAT.

Of the mammals so far examined this animal showed the developing stages of goblet-cells best. This was especially the case in young rats (6–8 weeks' rats were used). The initial stages of mucin-production could be observed in the cells towards the base of the crypts of Lieberkühn ; the mucin content of the cells increased as the crypt was followed towards the surface, but the goblet-cells on the surface showed a diminution.

Fig. 29 shows an impregnation of the basal cells of such a crypt by Cajal's method in which the position of the Golgi body with relationship to the nucleus is indicated. This preparation was obtained after fixation by Cajal's uranium-nitrate fixative injected intra-arterially, the tissue then being left in fixative for 7 hours. Impregnation follows best after a fixation of 6 to 7 hours, even when this is by immersion only.

Fig. 7 shows a cell with Golgi body impregnated by osmic acid. A comparison of the position occupied by the Golgi body and the position in which granules form in the immature goblet-cells shows that these coincide (Figs. 9 and 1). It might be asked why the granules and the Golgi body were not stained in the same preparation. The Golgi body of the gut epithelial cell is very compact, and the methods used by Nassonov to show secretion

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EXPLANATION OF PLATES.

FIG. 1.—Cat: Two cells from depth of a crypt of the colon 24 hours after exhaustion with dilute mustard oil. Fixation, "Susa." Iron-hæmotoxylin, muci-carmine, Metanil yellow. Though the granular form of the mucus has been destroyed by the fixative, the pink staining material is in the region occupied by the Golgi apparatus.

FIG. 2.—Further stage of development from the same cat.

FIG. 3.—Cat: Cell from crypt of colon 24 hours after exhaustion by dilute mustard oil. Fixation, intra-arterial formol-bichromate. The mucin granules are better preserved than in the previous cat. Their relationship to a clear area occupying the area of the Golgi apparatus is shown.

FIG. 4.—Cross-section of cell from same cat showing relationship of mucus granules to clear area surrounding them.

FIG. 5.—Dog: Cell from depth of crypt of small intestine "patch." Fixation, "Susa." The mucus appears only in the region of the Golgi apparatus.

FIG. 6.—Dog: Same preparation. Cell showing two zones of mucus.

FIG. 7.—Rat: Colon. Impregnation by Nassonov's method of the Golgi apparatus of a cell showing mucus formation. From the bottom of a crypt. Stained by neutral red.

FIG. 8.—Rat: Ditto. Shows displacement of Golgi apparatus towards the base of the cell by the collecting mucus.

FIG. 9.—Rat: Colon. Cell from base of crypt of colon. Shows formation of mucus granules in the neighbourhood of Golgi apparatus. In this specimen a clear area surrounding the granules is to be seen. Fixation, intra-arterial Nassonov. Carazzi's hæmatoxylin, and muci-carmine.

FIG. 10.—Rat: Same preparation. Cells from half-way up a crypt. Shows granules of mucus below main cup in relationship to clear areas. The neighbouring cell also exhibits these areas though no mucin is being produced. The nucleus and the protoplasm of the goblet stains more deeply than the epithelial cell. The attachment to the basement membrane is also smaller.

FIG. 11.—Rat: Same preparation. Cell from surface, showing narrow elongated cell. Granules of mucus in Golgi area. Upper portion discharging mucus.

FIG. 12.—Rat: Same preparation. Surface cells. Shows narrow cells with dark nuclei and protoplasm between larger cells with pale staining nuclei and protoplasm. Note cuticular border on both types of cell.

FIG. 13.—Cat: Cell from base of colonic crypt after application of dilute mustard oil (8 drops in 25 c.c. olive oil) for 5½ hours. Note presence of large numbers of filamentous mitochondria without any definite orientation. Nucleus omitted. Note flattening of cell. Fixation intra-arterial Nassonov. Heidenhain's iron-hæmatoxylin; muci-carmine.

FIG. 14.—Cat: Same preparation. Partially exhausted goblet-cell showing mitochondrial masses. It is to be emphasized that the mitochondria of the epithelial cells in the immediate vicinity were present as filaments.

FIG. 15.—Cat: Same preparation. Showing transition forms between large masses of mitochondrial substance and more filamentous forms. Epithelial cells with filamentous mitochondria on either side.

FIG. 16.—Cat: Same preparation. Many more filamentous forms present in partially exhausted goblet-cell.

FIG. 17.—Cat: Epithelial cell from towards base of crypt of colon after application of dilute mustard oil for 5½ hours. Shows the presence of abundant filamentous mitochondria. Fixation, intra-arterial Altmann. Heidenhain's iron-hæmatoxylin and muci-carmine.

FIG. 18.—Cat: Same preparation. From base of a crypt. Shows very long filamentous mitochondria met with and their relationship to the nucleus.

FIG. 19.—Cat: Same preparation. Exhausted goblet. Shows globular and filamentous forms of mitochondria. Note perfect preservation of mucin granules which occur in Golgi apparatus area only.

FIGS. 20 and 21.—Cat: Same preparation. Mitochondria more filamentous. Note mucin granules. In Figs. 19, 20 and 21 note that the area of the Golgi apparatus, though showing mucin granules, is free from mitochondria.

FIG. 22.—Cat: Normal. Developing goblet-cell at base of crypt. Note presence of filamentous mitochondria. Fixation intra-arterial Nassonov. Iron-hæmatoxylin; mucin unstained.

FIG. 23.—Cat: Same preparation. In immature goblet-cell commencing aggregation of mitochondrial substance into masses. Large goblet-cell shows protoplasmic network (stained black). The epithelial cells in this area all showed typical filamentous mitochondria.

FIG. 24.—Cat: Cells from base of colonic crypt. After mustard oil exhaustion. Note very large pale nuclei of pale epithelial cells, the dark staining of the protoplasm and nucleus of the smaller cell showing some mucin granules. Note spread "foot" of cell. Intra-arterial Nassonov. Iron-hæmatoxylin and muci-carmine.

FIG. 25.—Cat: Same preparation. Row of cells from crypt of colon. Shows the easily recognizable difference between the two types of cells. In Figs. 24 and 25 the mitochondria which were over-differentiated have been omitted.

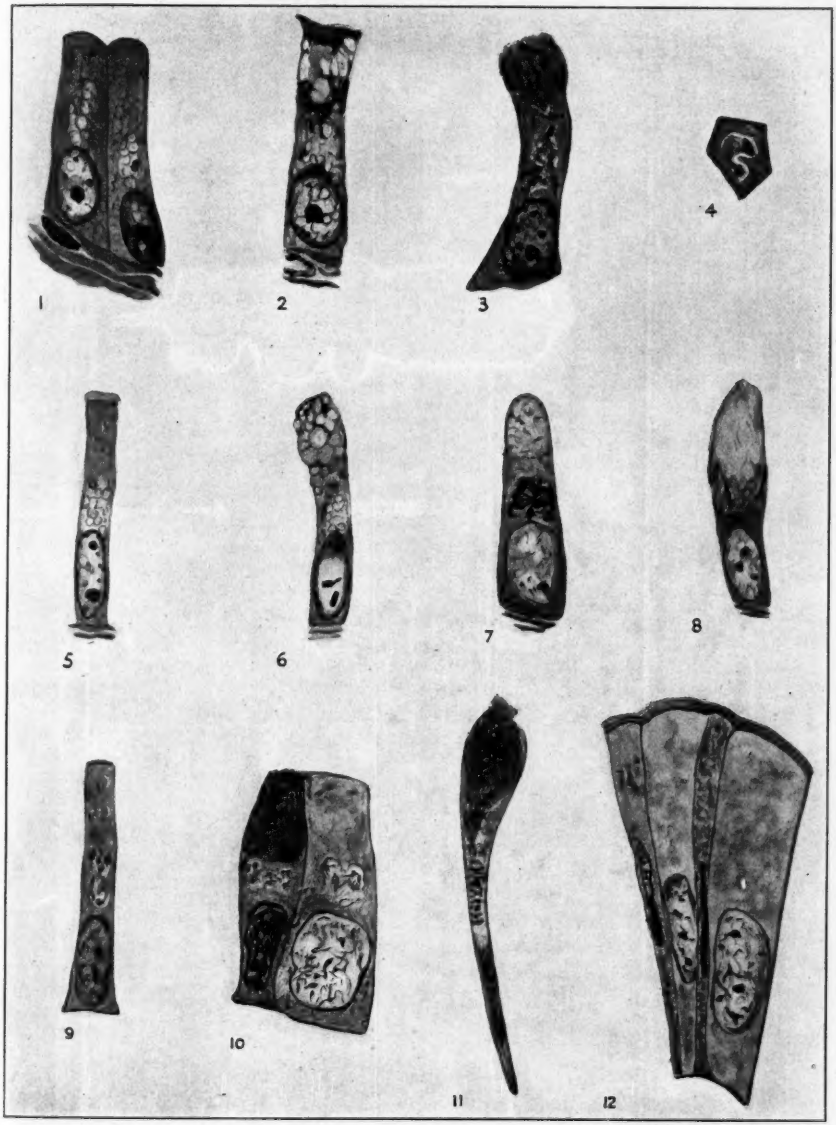
[Figs. 1, 2, 5, 6 and 8 by M. Collinet. With the exception of these, all drawn with Zeiss 1·5 mm. apochromat. 10 × eyepiece on this by camera lucida. The actual magnification is × 2000. as the drawing-board was 328 mm. below the eyepiece. I am indebted to Dr. Webb for permission to use this microscope, specially arranged to give very critical illumination, and for his advice.]

FIG. 26.—Cat: Exhaustion after mustard oil. Golgi apparatus impregnated by da Fano's method. Shows extreme flattening of cells, which nevertheless preserve a cap of argentophile material above the nucleus. × 510.

FIG. 27.—Formol fixed and iron-hæmatoxylin stained preparation from same cat, to show extreme flattening of epithelial cells. × 510.

FIG. 28.—From same cat. Shows Golgi apparatus in cells which have not become so flattened, but have a diminished protoplasmic content. × 1028.

FIG. 29.—Rat: Golgi body preparation by Cajal's uranium nitrate method.



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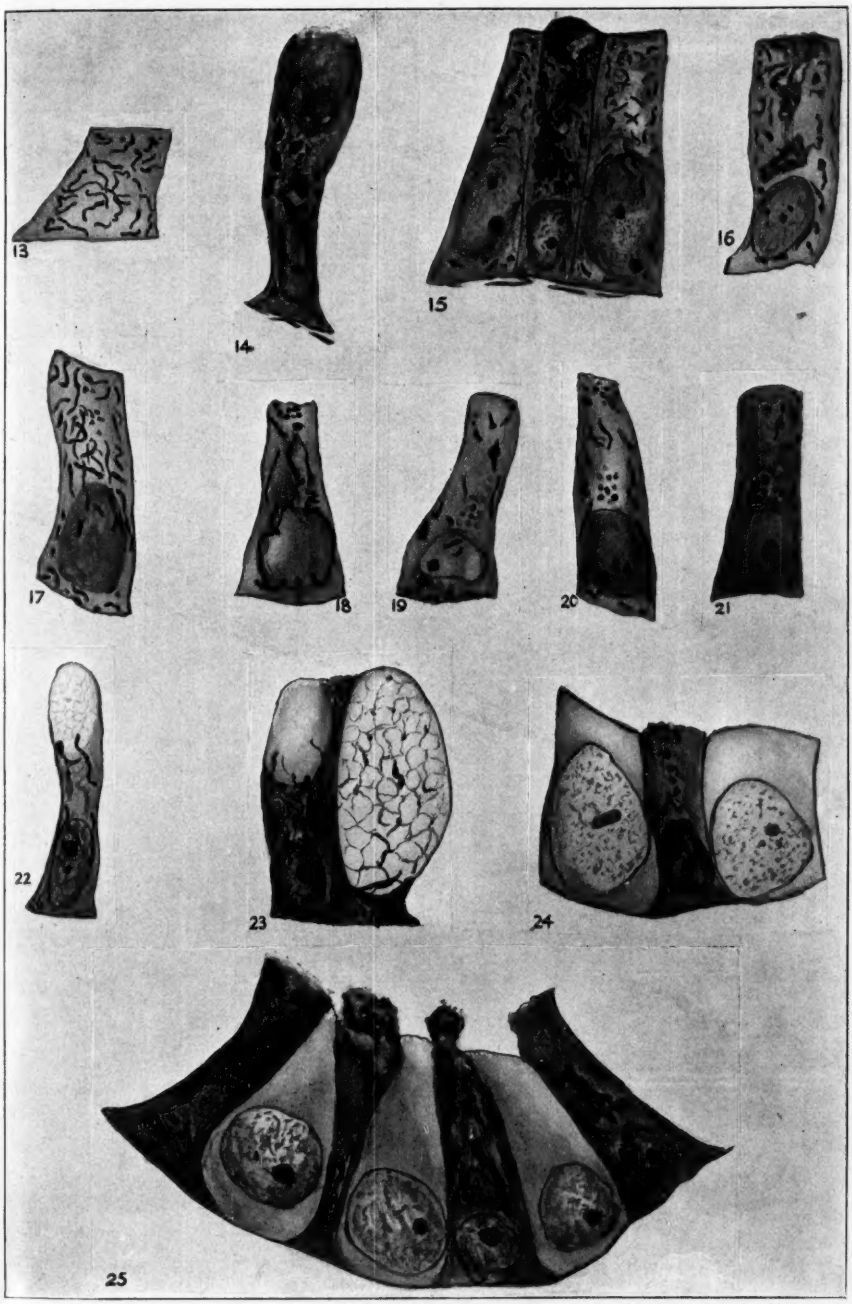

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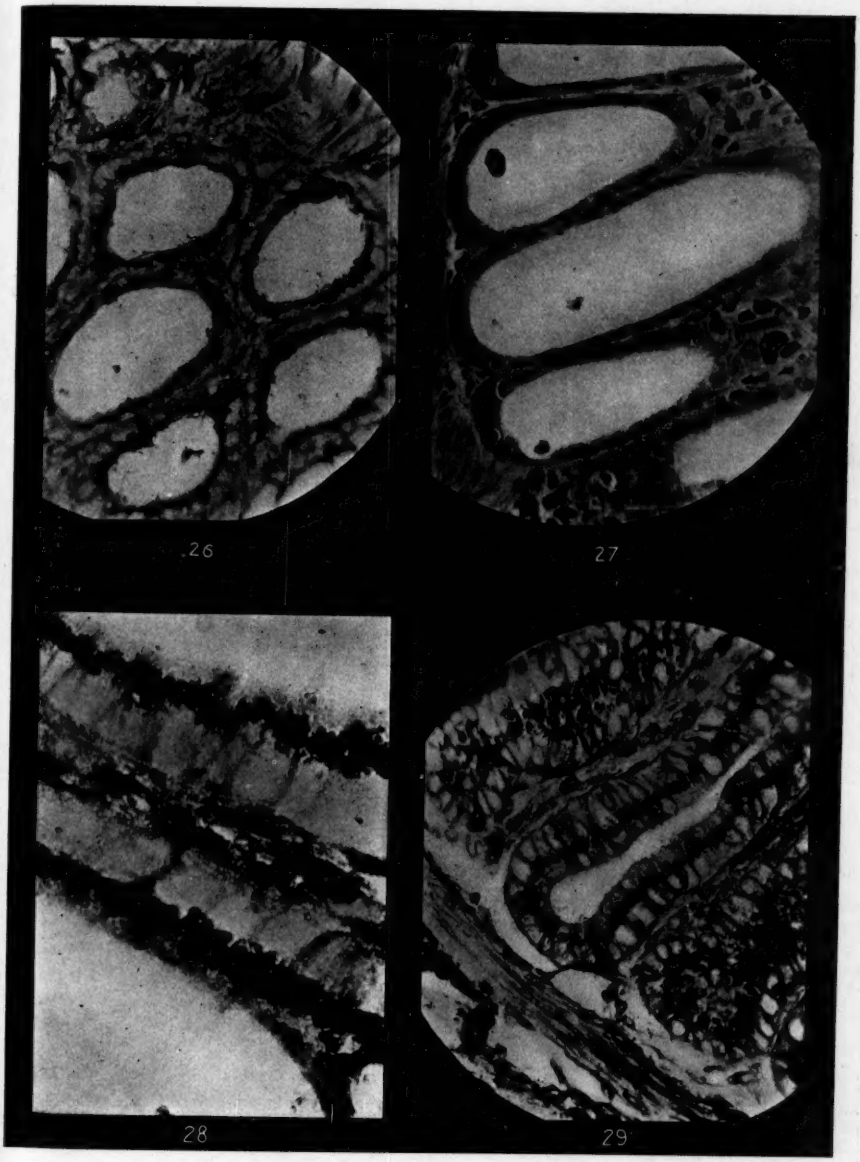

Florey.

granules have not proved effective. The specific mucin stains are useless after prolonged osmication, and though mucus will stain with muci-carmine after Cajal's technique, the resultant product is unsatisfactory owing to the compactness of the apparatus and the swelling of the mucus produced by the fixative. The method here employed of the comparison of two pictures is held to be valuable, though the precise relationship of the mucus granules to the impregnated substance has not been determined.

Fig. 10 shows granules of mucin occupying the Golgi area beneath the main cup of a fairly well-developed goblet. Fig. 11 shows one of the elongated cells from the surface of the colon. This is a discharging goblet in which a large part of the mucus has escaped, but granules are still present in the Golgi area. This series of drawings illustrates also that around the mucin droplets in the Golgi area there is a well-marked narrow pale zone, and it was noted in all cells examined that the mucin was contained in the light areas. This pale zone is also indicated in an ordinary epithelial cell in Fig. 10. It might be considered that the mucus has been shrunken away from the protoplasm or the protoplasm from the mucin to produce these light areas, but, from the fact that they also occur in ordinary epithelial cells, it seems reasonable to subscribe to the view that they represent a " negative " of the Golgi apparatus. If that is so, these pictures furnish good evidence of the close connection between the developing mucin and the Golgi apparatus. This series of figures also illustrates another fact observed with certainty only after fixation in Nassonov's fluid. At the base of the crypt the nuclei and protoplasm of ordinary epithelial cells and those producing mucin are generally indistinguishable by their staining reactions or size. As the crypt is ascended it is found that the cells containing mucus have more darkly staining nuclei and protoplasm. The nuclei also are more irregular in outline and smaller than those of the neighbouring cells. Some mucus-cells with dark-staining protoplasm and small nuclei are occasionally found towards the base of the crypts. On the surface the cells become very elongated, the mucus-cell being narrow, with dark nucleus and protoplasm. Cells possessing the same type of nucleus and protoplasm but no mucin are also found quite frequently here (Fig. 12). These latter also have a striated border like the paler staining epithelial cells.

According to Cajal (1914), during the development of a goblet-cell the Golgi body hypertrophies, and eventually fragments and disperses in the mucus. If, however, osmic acid impregnations are used, it can be seen that the osmiophile substance does not break up to be dispersed in the mucus, but is compressed towards the base of the cell (Fig. 8). It has not been possible to note the increase in Golgi substance described for other secreting cells during the process of elaboration of secretion. The osmic acid pictures, however, give no grounds for supposing that the Golgi substance is used up during the production of secretion, but rather that it is mechanically dislocated towards the base of the cell.

Mitochondrial preparations from intra-arterially Nassonov-fixed material do not give any evidence of the participation of the chondriome in mucin production. The filamentous mitochondria are distributed throughout the cell, beginning to elaborate mucus without any observable relationship to the area in which the mucus is first appearing.

DOG.

The method of inserting " patches " of colon into the abdominal wall has been described previously (Drury, Florey and Florey, 1929). The microscopical preparations here described were obtained from a similar patch of small intestine, taken from about the middle of the ileum. This patch had been inserted two years before the making of the histological preparation. A remarkable transformation had occurred in that the villi had disappeared, and there had been a very great increase in the number of goblet-cells. (This is more fully commented on elsewhere—Florey, Carleton and Wells, 1932.) At the bases of the long crypts of Lieberkühn, cut longitudinally, numerous cells could be found exhibiting the beginning of mucus formation. As in the case of the rat, the first appearance of stainable mucus is just above the nucleus in the area occupied by the Golgi body or vacuome (Fig. 5). In the crypts, stages can be traced in which there is a development here in this area, which appears to feed a collection of mucus in a small goblet-cup at the tip of the cell (Fig. 6).

The cells observed at the bases and, indeed, throughout the crypts differ from those in the rat and the cat (to be described) by being narrow and tall, both those exhibiting mucus production and those having the appearance of ordinary epithelial cells. The colon of the dog has not been examined in detail.

CAT.

In the normal colon of the cat the stages of development cannot be followed as in the two previous examples, because, though at the bases of the crypts the goblet-cells are very often smaller than those more towards the gut lumen, they almost always show mucus extending from the lumen border to the nucleus.

However, by the application of suitable dilutions of mustard oil, almost complete exhaustion of the mucus can be obtained (Florey and Webb, 1931). It was noted, in cats allowed to recover for 24 hours after a preliminary exhaustion, that mucus could be seen just above the nucleus and nowhere else. By the addition of metanil yellow to the staining, or by dark staining by Heidenhain's iron-hæmatoxylin, it is possible to see clearly in such material the development of mucus in relationship to clear spaces (vacuome). The vacuome is also clearly visible in the neighbouring epithelial cells. Cells showing this initial development only in relationship with the vacuome occur fairly frequently towards the base of the crypts, and, as in the rat, every stage can be followed up to the formation of a fully mature goblet (Figs. 2, 3, 4).

Further examination has shown that the same topographical relationship between the Golgi body and mucus droplets can be observed in the freshly exhausted colon. Figs. 19, 20, 21 are taken from a colon treated with dilute mustard oil (8 drops in 25 c.c. olive oil) for 5½ hours, and then fixed by intra-arterial injection of Altmann's fluid. In this preparation numerous cells towards the bottom of the crypts exhibit a few discrete spherical droplets of mucus just in the Golgi body area. Cells showing droplets in this position as well as mucus at the upper pole were also frequently seen. Similar appearances have been obtained in both Regaud- and Nassonov-fixed specimens. Gradations in mucus content were found from the few droplets above the

nucleus to the fully formed goblet-cell, but no cells were found with mucus only at the free border of the cell; there were always some droplets in the Golgi area.

In one particular experiment in which exhaustion of all goblet-cells was nearly complete it was possible to see definitely, about the middle of the crypts, that cells containing mucus had also a striated border. Mucus droplets were present in the Golgi body area, and in some cells there was a collection of droplets also just beneath the striated border. This position of the droplets was observed in many cells, and therefore it does not seem probable that confusion could arise from the superposition of an ordinary epithelial cell border just above the tip of the mucus-cell owing to obliquity of section.

In favourable preparations the striated border of the epithelial cells was very well seen on the cells towards the surface of the mucosa after prolonged treatment with mustard oil, so that the intense inflammation had not destroyed this striking feature. It is possible, however, to detect nuclear and protoplasmic differences between ordinary epithelial cells and exhausted goblet-cells after fixation in Nassonov's fluid, and less obviously, when visible at all, after the other fixatives employed.

The great majority of exhausted or partially exhausted goblet-cells are recognizable by their darker staining protoplasm and nuclei. The latter are also smaller and their outline is more irregular than those of the neighbouring epithelial cells. These cells may vary in size from the very narrow "ersatzzellen" type to cells which are as big as epithelial cells, all gradations being seen between them. They would appear to be cells all of one type though differing so much in size (Figs. 24, 25). Towards the base of the crypts, however, some cells containing mucus exist which do not exhibit these protoplasmic and nuclear peculiarities, and are indistinguishable from the ordinary epithelial cells except for their mucus content.

Another well-marked feature is the spreading "foot" of the exhausted goblet (Figs. 24, 25). The foot can be observed and has been frequently described in mature goblet-cells, but it is interesting to note that it is not caused by compression of the protoplasm towards the base of the cell by the accumulated mucus, because this peculiarity is retained even after evacuation.

Changes in the Golgi body.

Inasmuch as the present argument is based on the fact that the mucus droplets appear in the area comprised normally by the Golgi body, it is of some interest to follow the changes in this structure during the intense inflammation caused by mustard oil. Successful preparations have been made using ad Fano's cobalt nitrate method (Figs. 26 and 28 are taken from such a preparation). A control specimen from the same colon was fixed in formol-saline (Fig. 27). This control shows that extreme flattening of the epithelial cells had been produced at the base of the crypts, and that the cells showed no signs of desquamation. By comparison with Fig. 26, which is a silver impregnation, it can be seen that although the cells have apparently lost a considerable amount of their protoplasm, there is still a cap of argentophile material just above the nucleus.

Fig. 28 is a high-power photograph taken from portions of two crypts in

which the flattening of the epithelial cells was not so extreme. This again shows the presence of a cap of argentophile material in the usual place. This appears to have suffered no diminution in quantity from the normal. The cells at the base of the crypts have less argentophile material than these latter cells, so that presumably some has been lost together with the protoplasm. In some cells small pieces of impregnated substance occur at the cell border. These perhaps represent extrusion of Golgi material.

Mitochondrial Changes.

The behaviour of the mitochondria during the acute inflammation has also been studied, both in the goblet and epithelial cells.

Goblet-cells.—It is possible to pick out evacuated goblet-cells in a preparation stained for mitochondria by the fact that relatively large masses of stained material are usually present in them, in contradistinction to the filamentous type of mitochondria present in the surrounding cells. The presence of this latter form of mitochondria in cells immediately alongside mucus-cells is to be emphasized. Coupled with this fact is the constant occurrence in mucus-cells of these stainable masses, circumstances which preclude the possibility of the masses being caused by bad fixation. Their size is also against such a possibility. These masses have been stained by Heidenhain's iron-hæmatoxylin, Altmann's fuchsin and Benda's crystal violet. By all of these methods they give the same staining reaction as mitochondria, and there seems no reasonable doubt that they are the same substance.

It is common to find either discrete drops or elongated masses of mitochondrial substance appearing at the periphery of the main mucus mass in an emptying goblet. This arrangement can be well seen in cross-sections of such cells, the stainable portions lying between the membrane of the cell and the mucus. With further evacuation of the cell the masses tend to leave the periphery of the cell and to be dispersed in the partly revealed protoplasm (Figs. 14, 15, 19).

Stages of transition between these large masses and elongated rod-like mitochondria can be seen. Sometimes the rod appears to emerge from the mass; in other cases the mass, as it were, bestrides the rod. Mucus-cells are also found in which most of the chondriome exists as rods, traces only of the massive bodies being present (Figs. 16, 20, 21).

Figs. 22 and 23 are taken from the bottom of a crypt of a normal colon. They show the presence of filamentous mitochondria, most of which have become thickened, and may even have knobs on them. In the left-hand cell of Fig. 23 small masses of mitochondrial substance are present. It is again to be emphasized that the ordinary epithelial cells contained well-fixed filamentous mitochondria. A network staining black is illustrated in a mature goblet-cell in Fig. 23. Whether this network contains mitochondrial substance it is impossible to say, but this seems improbable, for when such a cell is partially evacuated the mitochondrial substance appears at the periphery of the cell.

Figs. 19, 20, 21 illustrate another constant appearance. There is no mitochondrial concentration in these cells in the region of the Golgi body; indeed, this area is particularly free. This observation would lend no support to the conclusion that mitochondrial substance is transformed into secretion by the

agency of the material of the Golgi-apparatus in the manner suggested by Parat (1928).

Epithelial cells.—These cells also exhibit changes in their mitochondrial content. In the most successful preparations made (Altmann-fixed) the epithelial cells of the surface had become crowded with fine short filamentous mitochondria, which formed almost a felt-work in the interior of the cells. These mitochondria were not oriented longitudinally, as is usually the case in these cells, but ran in all directions. They were particularly dense in the neighbourhood of the nucleus, forming a criss-cross mass completely surrounding it. The filaments were of the same size and shape in the infra- as in the supra-nuclear region. The disorientation of the mitochondria was notable throughout the thickness of the mucosa.

Cells in the base of the crypts were much flattened, and in some cases tended to be rounded. In these cells filamentous mitochondria were arranged symmetrically about the nucleus.

Fig. 13 illustrates such a cell. In this kind of cell there does not appear to be any particular condensation near the nucleus. These filaments are both longer and thicker than those in the cells of the surface. Fig. 17 illustrates the appearance of an epithelial cell which has not been very much flattened. There is no definite longitudinal orientation, and here again the mitochondria are less numerous, but thicker than in the surface cells.

Fig. 18 illustrates a condition only met with towards the bases of the crypts. Very long thick filaments are seen in close contact and extending round the nucleus. These thickened filaments were also present in the supra-nuclear protoplasm. Fig. 20, a mucus-producing cell, also shows the long mitochondria in contact with the nucleus. In one cell a thick filament was traced from the base almost to the upper pole of the cell—it was 13μ in length.

In some cells coiled and doubled-up mitochondria were seen, especially present in the subnuclear zone, where it is generally considered that they are granular.

The numbers of mitochondria are very much fewer in the basal cells than in those on the surface, which appear crammed with them.

RABBIT.

The colon of the rabbit has several features distinguishing it from those of the three previous species. The goblet-cells of the crypts when full of mucus are large and frequently pear-shaped, the smaller end being towards the lumen. The base of the cell is wide, and just above it lies the flattened nucleus. Among normal rabbits specimens are found which exhibit the vacuome very clearly. Within the light areas of the latter discrete droplets staining red with muci-carmine are found, large collections of mucus being in the upper pole of the cell. Corresponding to the epithelial cells of the cat are cells which present a net-like structure no matter what fixative be employed. This network stains deeply with muci-carmine, in some areas much more deeply than cells which are clearly goblet-cells. They are as a rule narrow cells, with narrow bases and small nuclei and larger free borders, which dovetail in between the goblet-cells. In some of them the net-like structure is deeply stained,

below the nucleus as well as above. These cells also stain intensely with Delafield's hæmatoxylin. The cells on the gut lumen surface are mainly epithelial cells with compact protoplasm, which fixes and stains like the epithelium of the other species (*i. e.* yellow, not red). The goblet-cells on the surface are much smaller than those in the crypts. They have the usual shape associated with goblet-cells, and most show small granules between the nucleus and the goblet-cup.

As in the cat, the goblet-cells can be evacuated by the application of dilute mustard oil. The reticulated cells of the crypts can only occasionally be recognized, the crypts being lined by cells with compact protoplasm, many of which show mucous remnants of the same kind as those exhibited by the cat.

THE GUINEA-PIG.

Some colons from this species were examined. They present the same features as those of rabbits. The reticulated or vacuolated cells of the crypts, however, are only slightly coloured by the muci-carmine. Here, again, after the application of an irritant these reticular cells are no longer apparent, their place being taken by cells with compact protoplasm.

DISCUSSION AND SUMMARY.

Evidence has been produced from the rat, cat, dog and rabbit that mucus granules are formed in intestinal cells in the region of the Golgi body. This is quite a constant finding in different preparations, so that the deduction may be made that the granules are formed in relationship with the vacuome or Golgi body, thus supporting the conclusion of Nassonov, Bowen and others for other gland-cells. No evidence has been obtained that the Golgi material is " used up " in elaborating the secretion; rather is it dislocated towards the base of the cell owing to the accumulation of secretion products. In reference to the term " vacuome," it is not the author's intention to discuss the various theories associated with this name. There can be no question, however, that it is possible to observe in suitably fixed material clear areas occupying the Golgi body position. The author subscribes to the view that these light areas correspond to the material blackened by osmium or silver, without offering an opinion as to the exact mode of impregnation by these metallic reagents.

The intense stimulation of dilute mustard oil does not cause the disintegration and dispersal of the Golgi apparatus from its usual position, except at the extreme bases of the crypts.

Some light on the question as to whether ordinary epithelial cells can become goblets is given by these observations. The following interpretation of the findings is given: Towards the bases of the crypts an indifferent type of cell exists; some of them start to elaborate mucus in the region of the Golgi body. These mucus-cells are indistinguishable histologically from the epithelial cells except by their mucus, as is clearly seen in both the rat and the cat. During the maturation of these cells they pass towards the lumen of the gut (Bizzozero's theory), and it is then that the protoplasm and nuclear differences here described appear. These differences, once acquired, persist, as is seen in the surface epithelium of the rat's colon and the artificially exhausted goblet-cells

of the cat. On the evidence from these preparations it is not considered probable that the goblet-cells revert to cells completely indistinguishable from the epithelial cells, though they may acquire a striated border. In other words, goblet-cells have become distinct entities, although starting from an indifferent cell.

The mitochondrial differences are marked between the goblet-cells in the exhausted cat colon and the epithelial cells, but this is not perhaps a permanently distinctive feature, because the massive mitochondria apparently re-form into filaments. In the rat no difference was seen in the mitochondrial content of the two types of young cells.

With regard to another disputed point, evidence has been obtained. The question has been long debated whether a goblet-cell, once having been evacuated, can elaborate a further amount of mucus. In a freshly stimulated colon goblet-cells can be found which are clearly almost completely exhausted of their previous content, but in which granules are present just above the nucleus. This same division into a zone of granules just above the nucleus and a discharging tip of the cell is well seen in the normal rat colon and rabbit colon. A reasonable interpretation in these cases is that the mucus-cell is elaborating a further supply of mucus during discharge. This view is strongly reinforced by experiments in which the animal was allowed to recover for 24 hours after mustard oil exhaustion.

The conclusion is reached that a goblet-cell may be manufacturing mucus in the Golgi area at the same time as it is being discharged at its apical border.

From the mitochondrial preparations no support has been obtained for the view that the mitochondria participate in mucus elaboration. From the fact that in the rapidly exhausted goblet-cell mitochondrial substance reappears in large masses, it would seem probable that during the filling of the cell with mucus the chondriome is compressed together so that the mitochondria coalesce. With the relief of pressure these masses are capable of reassuming filamentous form in that transition stages can be seen. The interpretation of the other mitochondrial changes is more difficult. Perhaps they represent an increased production and altered orientation under the inflammatory stimulus.

I am greatly indebted to Prof. P. Bouin for the hospitality of his laboratory and for advice. I wish also to thank Dr. P. Benoît for showing me various technical procedures. I am indebted also to the Government Grants Committee of the Royal Society for a grant towards the expenses of this work.

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SCHICK TESTS IN ICELAND.

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Received for publication July 11th, 1932.

As diphtheria has been practically unknown, not only in Reykjavik, but in Iceland as a whole during the last six years, and comparatively rare for some years before that time, an investigation into the immunity of the population was thought desirable. Such an investigation might be expected to throw some light upon the cause of this greater decrease in diphtheria morbidity, whether it is to be attributed to vanishing sources of infection, or mild, latent infections masked as simple sore throats. Further, the investigations in Greenland by Heinbecker and Irvine-Jones (1928) and Bay-Schmidt (1925; 1929) tended to throw some doubt upon the value of the Schick test, and this was another reason why it seemed desirable to have Schick tests made in another isolated population, where the conditions were relatively simple and diphtheria practically unknown for a number of years.

Material.

Young children from 2 years and upwards would have been the material of choice for these investigations, but owing to the difficulty of gathering great numbers of young children from their homes for this purpose, it was decided to make all the tests on children in the elementary schools. There we had children of from 8–14 years, most of them 8–13 years of age. Altogether 814 children have been tested.

The necessary materials for the Schick test were supplied by the Statens Seruminstitut, Copenhagen, and this opportunity is taken of expressing our gratitude to the Seruminstitut for having provided these materials free of charge.

Freshly made dilutions of toxin were invariably employed, and 1/10 c.c. of the diluted toxin was injected intra-cutaneously into the left forearm, and the same amount of the heated toxin into the corresponding place on the right arm. The first 456 tests, made in December, 1931, were given three readings each, *i. e.* after 2, 4 and 6 days respectively, but after that the first reading was omitted as it seemed unnecessary. Very few pseudo-reactions were noted, and these have been omitted from our figures.

The results are given in Table I.

It will be seen that out of 814 children, 657, or 81·9 per cent., gave positive reactions, and only 18·1 per cent. were Schick-negative, the percentage of negatives being higher in males than in females, as the majority of workers have found. The increase in negatives with advancing age is well marked. Girls are practically all positive at 8 years, but from this age upwards the immunity is steadily increasing, until about 30 per cent. of the girls and 40 per cent. of the boys are negative at 12 years.

TABLE I.—*Schick Reaction in Reykjavik Children 8–14 Years Old.*

Age.	Schick-positives.						Schick-negatives.					
	Females.		Males.		Both sexes.		Females.		Males.		Both sexes.	
	Number.	Percentage.	Number.	Percentage.	Number.	Percentage.	Number.	Percentage.	Number.	Percentage.	Number.	Percentage.
8 years	39	92·86	78	82·98	117	86·03	3	7·14	16	17·02	19	13·97
9 ,,	68	90·87	55	79·71	123	85·42	7	9·33	14	20·29	21	14·58
10 ,,	62	86·11	71	82·56	133	84·18	10	13·09	15	17·44	25	15·82
11 ,,	58	84·06	62	81·58	120	82·76	11	15·94	14	18·42	25	17·24
12 ,,	57	79·17	38	66·67	95	73·64	15	20·83	19	33·33	34	26·36
13 ,,	41	70·69	24	60·00	65	62·50	17	29·31	16	40·00	33	37·50
14 ,,	3	..	1	..	4	..	3	..	..	..	..	..
Total	328	83·25	329	77·78	657	81·92	66	16·75	94	22·22	157	18·08

Comparison with Other Countries.

The greatest number of Schick tests seem to have been made in America, where Zingher (1923) has collected the results of more than 150,000 Schick tests on American children. His numbers are given below, with the corresponding percentages in Reykjavik for comparison.

Percentage of Schick Negatives.

Age.	America (Zingher).	Reykjavik.
1–3 years	16·8	..
5–6 ,,	39·7	..
7 ,,	49·6	..
8 ,,	56·5	13·97
9 ,,	63·5	14·58
10 ,,	67·8	15·82
11 ,,	70·7	17·24
12 ,,	71·8	26·36
13 ,,	73·4	37·50
14 ,,	76·9	..
15 ,,	80·3	..
16 ,,	81·8	..

The difference is conspicuous, and in two respects: First the susceptibility to diphtheria seems to be much more widespread in Reykjavik, where 8-year-old children are as susceptible as American children of 1–3 years. It must be kept in mind, however, that Zingher's figures are average numbers from many different places. In some rural districts up to 85–90 per cent. were found Schick-positive, and in the same city a difference was noted between different social strata. Children from the poor classes showed a higher percentage of negatives than children in private schools from homes in better economic circumstances, where exposure to infection is less likely to occur. Our material in Reykjavik comprised children from all sorts of homes; the result, therefore, points clearly to a low rate of diphtheria infections in Reykjavik.

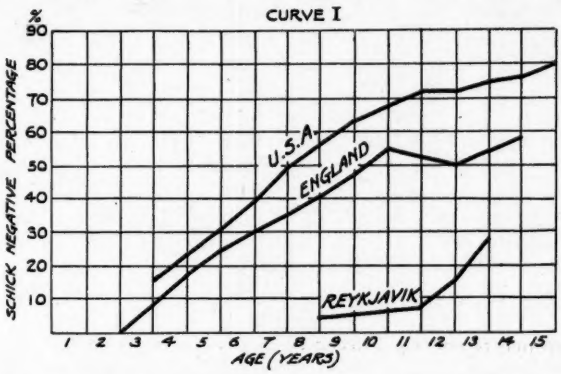
But there is also another striking difference to be noted in this comparison. The findings of Zingher in America show the percentage of immunes rising steadily from year to year, the rise being rapid during the first few years of life, and from then on more slowly. In Reykjavik, on the contrary, the number of immunes only increases 2 per cent. from 8–12 years, and then increases rapidly. Curve 1 shows the results of Schick tests at different ages in U.S.A., England and Reykjavik, the figures for England being those of Bousfield (1929).

In America and England the numbers of negatives increase steadily with advancing age, being considerably higher than the corresponding numbers for Reykjavik.

But an interesting feature of the curve for Reykjavik is the comparatively steep rise which occurs after the twelfth year. What is the cause of this?

Is it to be explained by a physiological increase in natural immunity at this age, or by exposure to infection from which the younger age-groups have been spared?

Kellogg and Stevens (1927), from the estimation of antitoxin in the serum of Schick-negative individuals, conclude that immunity to diphtheria may be physiological, and need not be acquired through infections. Rosling's (1928) investigations of immunity to diphtheria in Copenhagen suggest that the Schick test is of restricted value, and point to the influence of hereditary and physiological factors in diphtheria immunity. Bay-Schmidt (1925; 1929), who has made Schick tests in isolated Eskimo populations in Greenland, finds a sudden rise in Schick-negatives after the age of 15 in both sexes, and from



this concludes that diphtheria immunity is a concomitant of puberty, the inherited immunity of the newborn being gradually lost during the first years of life.

Heinbecker and Irvine-Jones (1928) found 37 out of 49 individuals in Greenland Schick-negative in a population where there was no record of diphtheria. Dudley (1929), however, is of the opinion that the negative reaction is the result of an antigenic stimulus.

In this connection it is of interest to note the occurrence of diphtheria in Reykjavik during the past 15 years. Our Public Health Reports give the following figures:

Year.	Number of cases.	Attack rate per 1000 of population.
1917	37	..
1918	16	..
1919	8	..
1920	69	3·300
1921	126	6·900
1922	45	2·300
1923	49	2·400
1924	37	1·300
1925	12	0·500
1926	50	2·200
1927	2	0·080
1928	2	0·080
1929	4	0·015
1930	2	0·070

The years 1920 and 1921 show the highest rates, a slight wave of the disease going over the town in 1921, *i. e.* 10 years before the tests in question were made. Since children under 2 years are less exposed to infection than older ones, it would be amongst the latter that a response to the antigenic stimulus provided by the wave of disease of 1921 would be expected, and the increase in immunity in the age-groups who had been exposed to the 1921 epidemic favours the opinion that the increase in percentage of negative reactors among the children of 12 years and over in this isolated population is not caused merely by physiological changes, but is dependent on infection with the diphtheria bacillus.

SUMMARY.

Of 814 children in Reykjavik between the ages of 8 and 14, 81·9 per cent. gave a positive Schick reaction.

Up to the age of 12 there was only a slight increase in the percentage of negative reactions, thereafter the percentage showed a sharp rise. Since diphtheria has been practically unknown in Reykjavik during the last six years, and rare previously to that with the exception of 1920–1921—an epidemic wave occurring in 1921—it is suggested that the increase in reactors of 12 years and over is explained by the wave of diphtheria ten years ago.

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GROWTH AND METABOLISM OF THE BOVINE PLEURO-PNEUMONIA VIRUS.

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Received for publication July 15th, 1932.

A great bar to a more precise knowledge of most filterable viruses is that so far the only universally accepted method for determining their presence or estimating their concentration is by their pathogenic effect upon animals. Loss of pathogenicity by a virus growing in culture would be very likely to be described as the death of the virus. The work of Mr. J. E. Barnard

demonstrating the presence of non pathogenic viruses growing in ordinary broth and serum cultures must at the minute remain unconfirmed by other methods.

Some approach may possibly be made to the problem by a study of the metabolic processes of the viruses. Should these prove capable of estimation, the presence of a virus might be detected by changes taking place in a medium, and multiplication of a virus be discovered by a corresponding increase in metabolism. This should certainly be possible in the few cases where the presence of living tissue is not necessary for growth in culture—for instance with the small saprophytic organisms described by Mr. Barnard. Whether it will prove possible in the case of the pathogenic viruses may depend on the extent to which their metabolism differs from that of the tissue in which they are growing.

As a beginning it was obviously necessary to work with a virus which undoubtedly grows in culture, and we have therefore used the filter-passing organism of bovine pleuro-pneumonia.* This is one of the very few viruses which will grow in broth and serum (5%–10% of heated serum is required) without the presence of tissue. The virus becomes non-pathogenic after repeated subculture, but makes a visible turbidity in the medium so that its growth can be seen.

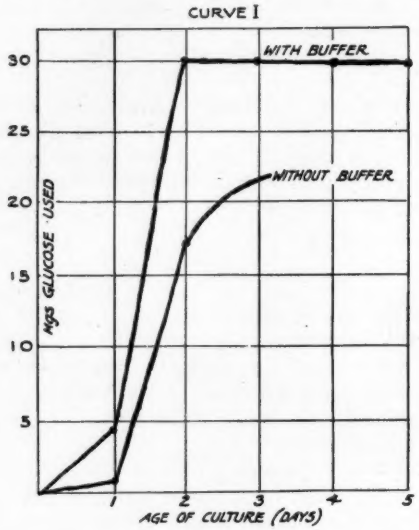
FERMENTATION OF GLUCOSE

It was known that pleuro-pneumonia fermented glucose and some other sugars (Walker, 1930), and we decided to make a quantitative study of this glucose breakdown, both to get an idea of the metabolic activity of these very small organisms, and to see if we could obtain evidence of increasing activity suggesting growth of the organism. Part of the reducing substance present in ordinary meat broth is fermentable by the virus, but the amount is small, and it was not possible to obtain significant results without the addition of fresh glucose. We made use of a meat broth which had been freed from glucose during storage by an accidental bacterial contamination, and had subsequently been filtered and steamed. This broth contained no reducing substance capable of being fermented by pleuro-pneumonia, and glucose and phosphate buffer could be added to it in the required amounts. The glucose was always sterilized by filtration, as it had previously been found that glucose which had been sterilized by steaming had properties very different from those of the fresh sugar (Watchorn and Holmes, 1931). Tubes containing 20 c.c. of broth and 2 c.c. of serum were used, with amounts of glucose of 30 to 40 mgm. added, and usually phosphate buffer as well. Heavy inoculations were used throughout these experiments, two drops of a three-day culture being added for each 5 c.c. of broth. Samples were taken immediately after inoculation and on each subsequent day. It was always found that during the first day little or no sugar disappeared, whereas during the second day about 30 mgm. were broken down. After this there was no further breakdown, all added

* *Note.*—Although there is some dispute as to the true nature of this organism, no forms other than spherules and small granules have been seen in liquid media such as are used in the following experiments.

glucose having apparently been utilized. Curve 1 shows examples of glucose breakdown with and without phosphate buffer.

The very great increase of glucose utilization during the second 24 hours as compared with the first 24 hours of culture certainly suggests an increase in the amount of virus present, but it must be remembered that delay in fermentation might be caused by a lack of an activator or a co-enzyme in the broth, which might then be made by the virus itself during the first 24 hours. If this objection is correct it should not be possible for washed virus to ferment sugar. Experiments testing this point are in progress. In the case of this organism there is very little doubt that considerable growth has occurred



during the second day, as increase in the turbidity of the culture can be observed, but since it is hoped to apply these methods to other filter-passing organisms, it is obviously desirable to obtain further confirmation of growth.

OXIDATIVE MECHANISMS.

From its behaviour in culture it has for some time been assumed that the organism was definitely aërobic, and it therefore seemed likely that an uptake of oxygen or reduction of methylene-blue might be demonstrated. We did, in fact, find that methylene-blue added to a culture of the bovine pleuro-pneumonia virus (pH adjusted to 7·4) was fairly rapidly reduced.

By centrifuging at about 3000 revolutions for 15 minutes a small precipitate can be made to settle out of a two- or three-day culture. This precipitate contains some virus, but in all probability some non-living substances from the medium as well. When re-suspended in saline or Ringer's solution this precipitate will reduce methylene-blue at 37° C. very slowly,

taking about 1 hour to reduce 0·2 c.c. of M/1000 solution. In the presence of broth or sodium lactate, however, this reduction is very rapid, about 8 minutes being enough to reduce the same quantity of methylene-blue.

Lactic acid is the only hydrogen donator which has so far been identified. Glucose, formate, succinate, alanine and hypoxanthine have little if any effect on the reduction-time. The activity of the virus in dehydrogenating lactic acid is very considerable, an amount of centrifugate with the dry weight of 1·5 mgm. reducing 0·2 c.c. of M/1000 methylene-blue in 5 minutes in the presence of sodium lactate (pH 7·4). It is very probable that this centrifugate does not consist entirely of virus, and that the activity of the virus is thus even greater than it appears to be from this figure.

It is obvious that these facts can be made use of in constructing a growth curve for the organism, since an increase of lactic dehydrogenase in the culture must almost certainly mean an increase in the virus which brings about the dehydrogenation.

Twenty c.c. of broth and serum were sown with 8 drops of a three-day culture, and samples were taken immediately after inoculation and on every subsequent day. The samples were all kept under sterile conditions in the ice-chest until the end of the period of growth. The pH of each sample was then adjusted to 7·4 and the same amount of sodium lactate added to each; 2 c.c. of each sample was then placed in a vacuum tube with 0·2 c.c. methylene-blue, the tubes evacuated, and the reduction-time of each tube recorded. The reduction-time was about 5–5½ hours at the beginning of the experiment, and as little as 10 minutes on the third day. After the third day of growth in ordinary meat broth the dehydrogenase activity showed a slow fall, this agreeing with what was already known from bacteriological experience of this strain. It was known that the best time for subculturing is on the third day.

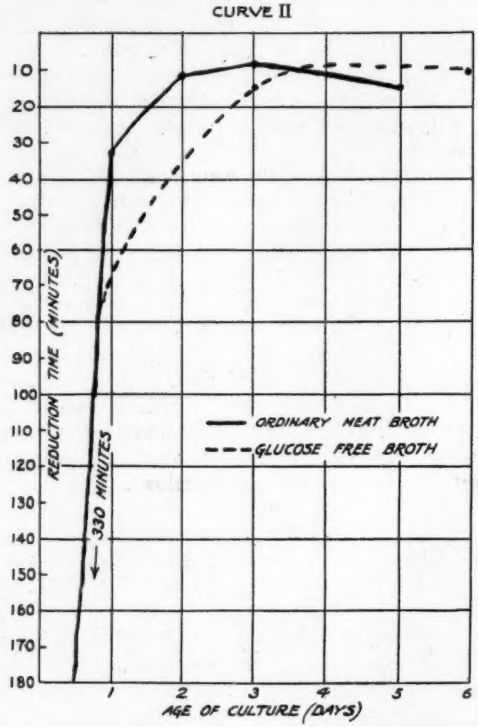
Two typical sets of figures are given below:

TABLE I.

Age of culture in days.	Reduction time in minutes. Experiment 1.	Experiment 2.
0	About 300–330	330
1	25	33
2	..	12
3	10	10
4	..	..
5	16	15

When setting out these data in the form of curves we have used two ways of expressing them. In Curve 2 we have plotted the time in minutes reversed against the number of days in culture; this method perhaps gives the best picture of the actual growth-rate of the virus, with the very large multiplication on the first day. The later part of the curve is better shown by the second method (Curve 3), in which the amount of lactic dehydrogenase present is given in units. The amount present immediately after sowing is taken as one unit, the reduction-time being then about 5 hours; any amount which reduces in one-tenth of that time is called ten units and so on.

It cannot, of course, be claimed that this method gives a quantitatively accurate estimate of growth. The curve must certainly be unduly flattened, for instance, since we know from experiment that a fourfold increase in virus means about a third and not a quarter of the reduction-time. To avoid this error it would be necessary to dilute all samples until they gave the same reduction-time, which would entail much waste of material. The shape of the curve may also be distorted by the presence of other hydrogen donators in the

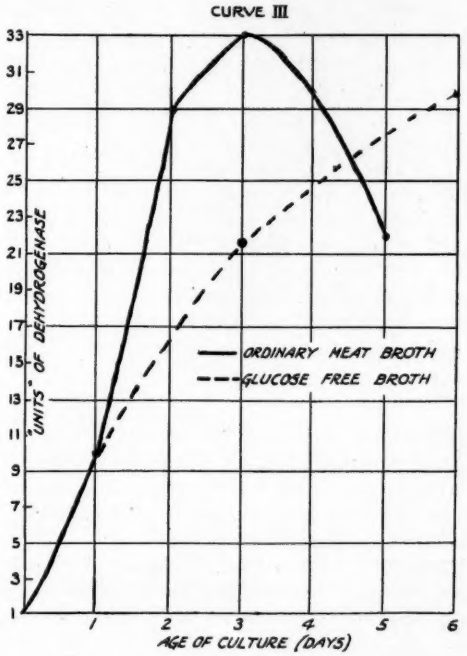


broth which become exhausted during growth. We have some grounds for believing that an unknown donator is present in ordinary meat broth, and that this is all removed by the virus before the third day. Since we cannot supply this donator as we supply the lactate, the reduction-time on the third day when compared with that on the first day must be relatively too slow. However, in the case of the glucose-free broth it seems that lactate is the only donator present, and this error is removed.

In the glucose-free medium (Curves 2 and 3) the amount of dehydrogenase present does not fall off after the third day. Reasons for that are discussed later.

NITROGEN METABOLISM.

For purposes of estimating ammonia production by the virus, it was necessary to grow the culture in a broth which had been treated with "permutit" to remove the rather large amounts of ammonia already present. The broth was acidified (pH 6·0), passed through a thick layer of permutit on a glass filter and neutralized again before use. Growth in this medium was satisfactory, but only small and rather inconstant amounts of ammonia were produced, and these only at the beginning of growth; much importance



cannot be attached to this at present. It is possible that if the necessary precursor could be found, the pleuro-pneumonia virus would produce larger amounts of ammonia, but obviously none of the substances present in meat broth are readily deaminated by it.

There is no appreciable alteration in the number of free amino groups in the broth during the course of growth. The estimations were made by formol titration to pH 7·3, using a comparator.

It must be remembered that whereas the energy requirements of a small growing organism are probably large, the actual amounts of protein and other materials required for building up the cell substance are very small. The pleuro-pneumonia virus seems to obtain its energy by the breakdown of carbohydrate and lactic acid rather than protein and amino acid.

REACTION.

It was known that an acid reaction is unfavourable to the growth of pleuro-pneumonia virus (Medical Research Council's 'System of Bacteriology, Part 7), and we are inclined to think that the falling off in the amount of live virus present after three days in culture in ordinary broth is due to the development of an acid reaction. On the sugar-free broth the culture remains at a neutral pH, and the amount of live virus present, as judged by the lactic dehydrogenase activity, is as high at the end of six days as at the end of three days. The small amount of fermentable reducing substance present in ordinary meat broth probably accounts for the formation of acid. When extra amounts of glucose are added death is far more rapid unless sufficient buffering solution is added at the same time. If 0·2 per cent. glucose is added without phosphate or other buffer there is very little lactic dehydrogenase left in the culture at the end of three days, and the glucose fermentation may also be slower. It is stated in the Medical Research Council's 'System of Bacteriology' that better growth is obtained in the presence of 0·5 per cent. glucose. It may be true that growth would be more rapid, but as far as we can judge the actual amount of virus left alive after 48 hours is likely to be less if considerable amounts of glucose are present than in ordinary meat-broth or even glucose-free broth. The growth on glucose-free broth is on the whole a little slower than on ordinary broth containing a small amount of fermentable reducing substance. It seems that, in the case of our media, there is little or no growth if the pH is as low as 6·4, and fairly rapid death of the virus if it falls much below 6·0.

SUMMARY.

An attempt has been made to estimate the growth of the filter-passing organism of bovine pleuro-pneumonia by estimating its metabolism.

There is little or no production of ammonia in cultures, and no perceptible increase in amino-nitrogen (formol titration).

There is a very slight fermentation of glucose during the first 24 hours of culture and a very large fermentation during the second 24 hours. This increase of metabolic activity probably indicates increase in the amount of organism present.

The pleuro-pneumonia virus can reduce methylene-blue in the presence of sodium lactate. The growth of the virus in culture can thus be conveniently estimated by following the increase in lactic dehydrogenase in the culture.

No hydrogen donators other than lactate have been identified.

We have to thank the Medical Research Council for personal grants.

We are glad to have he opportunity of thanking Dr. Laidlaw and Mr. Barnard for cultures and much other help.

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A SEROLOGICAL STUDY OF *V. CHOLERÆ* AND RELATED VIBRIOS.

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Received for publication July 15th, 1932.

THIS paper gives the results of an attempt to classify serologically some 45 strains of cholera, paracholera or cholera-like vibrios isolated at different places and at different times.

With the newer knowledge of the antigenic composition of bacteria, it had become necessary to review the old serological distinctions and relationships inside the group of cholera and cholera-like vibrios.

Of the 45 strains, 35 gave the typical biochemical reactions of cholera; they fermented glucose, maltose, mannitol and saccharose and gave the cholera red reaction; of these 35 biochemically typical strains, 23 were non-hæmolytic and 12 were hæmolytic to sheep red cells.

The remaining 10 strains, giving atypical biochemical reactions, were some of them non-hæmolytic and some hæmolytic.

Biochemically Typical, Non-hæmolytic Strains.

Serial number in the National Collection of Type Cultures, London.	Date of deposition or isolation.	Source.
17	Isolated 1914	Russia.
23	Deposited 1920	Straits Settlements.
27	,, 1920	Petrograd.
29	,, 1920	Bombay.
689	,, 1920	,,
717	Isolated 1917	,,
1633	,, 1922	India.
2463	,, 1922	Pekin.
2899	,, 1926	U.S.S.R.
2952	Deposited 1929	Bombay.
3241	Isolated 1922	U.S.S.R.
3242	,, 1922	,,
3243	,, 1923	Taschkent.
3636	,, 1929	India (healthy individual).
3637	,, 1932	India.
3638	,, 1932	,,
3639	,, 1932	,,
3640	,, 1932	,,
3641	,, 1932	,,
3642	,, 1932	,,
3643	,, 1932	,,
3644	,, 1932	,,
3645	,, 1932	,,

Biochemically Typical Hæmolytic Strains.

Serial number in the National Collection of Type Cultures, London.	Date of deposition or isolation.		Source.
30	Isolated	1916	Montazah (fæces).
685	Deposited	1920	Bombay (sewerage).
686	,,	1920	Bombay.
718	Isolated	1917	,,
721	,,	1920	Bombay (water).
2890	,,	1905	El Tor.
3409	Deposited	1931	Saigon.
3646	Isolated	1929	India (patient).
3647	,,	1929	,, (water).
3648	,,	1929	,, (,,).
3649	,,	1929	,, (,,).
3650	,,	1929	,, (patient).

Biochemically Atypical Non-hæmolytic Strains.

Serial number in the National Collection of Type Cultures, London.	Date of deposition or isolation.		Source.
722	Isolated	1918	Bombay
723	,,	1918	,,
2084	,,	1925	Dorsetshire.
3651	,,	1929	India (water).
3652	,,	1929	India (healthy individual).

Biochemically Atypical Hæmolytic Strains,

Serial number in the National Collection of Type Cultures, London.	Date of deposition or isolation.		Source.
559	Isolated	1916	Egypt.
1548	Deposited	1923	Alexandria.
2902	Isolated	1922	Archangel.
3653	,,	1929	India (healthy individual).
3654	,,	1929	India (patient).

Of these 45 strains all except one (717) were quite salt stable and therefore, according to this test, to be regarded as smooth. For lack of sufficient data no attempt has been made to correlate the strain and the type of illness, if any, with which it was associated. To obtain such correlation it would be necessary to work in direct connection with a cholera epidemic, and when the typical antigens of true *V. choleræ* have already been established. Only then will one be in a position to decide to what extent serologically aberrant forms are responsible for disease of a choleraic nature.

TECHNICAL METHODS.

Hæmolysis.

The hæmolysis test was carried out according to Greig's method (1914). The cultures were grown in alkaline broth for 3 days at 37° C. ; 1 c.c., 0·5 c.c., 0·1 c.c. and 0·05 c.c. quantities were taken, brought where necessary to 1 c.c. with saline and added to 1 c.c. of 5 per cent. sheep red blood-corpuscles in saline (Greig recommends goat red blood-corpuscles). The mixtures were incubated at 37° C. for 2 hours and kept in the cold room overnight, the result being read the following morning.

Preparation of Flagellar Suspensions.

For animal inoculation and for agglutination purposes a concentrated flagellar suspension was prepared according to a slight modification of the method adopted by Balteanu (1926). The growth on a Roux bottle incubated at 37° C. for 24 hours was washed off with 25 c.c. saline, shaken for 2 hours and centrifuged at moderately high speed for one hour. To the water-clear supernatant fluid, rich in flagella, was added a suspension of the whole organism to give an opacity equivalent to about a thousand million organisms per c.c. The use of such concentrated flagellar suspensions quite definitely increases the proportion of strains which will agglutinate with cholera serum. The strains in question are obviously deficient in H antigen, and only upon concentration is their relationship to the serum demonstrated.

Antisera were prepared in rabbits by the intravenous injection at weekly intervals of such suspensions, heated to 60° C. for one hour, the initial dose being 0·5 c.c. and increasing by 0·5 c.c. amounts. We had hoped to obtain good H and O titres after a series of 4 inoculations, but even after as many as 8 injections the O titre was often no higher than 1/400, and only in a few instances as high as 1/1600 ; the H titre on the other hand frequently reached 1/12800.

For the absorption of sera with H titres as high as this, and especially when such a sensitive emulsion as the concentrated flagellar suspension was used for agglutination, it was found that at least 2 slopes were needed for every c.c. of 1/100 dilution. The absorbing mixtures were left at 37° C. for 2 hours before centrifugation.

For agglutination the usual serial dilution of serum was adopted, starting with 1/100, and the result was read after 2 hours' incubation in a water-bath at 50° C. for the flagellar agglutination, and after 4 hours' incubation at the same temperature and on the bench overnight for the somatic agglutination, for which an ordinary steamed suspension of the organism was used.

Eleven strains were selected from the collection, and anti-sera prepared by the inoculation of rabbits as described above. As a fairly potent somatic titre was obtained with only 5 of the strains, it was with these that somatic relationships were established.

Cross agglutination between the whole set of strains and these 11 sera showed at once that there was considerable serological overlapping between the various biological groups, and that this was particularly noticeable with flagellar agglutination.

If one takes the serum prepared with the typical cholera strain 23 as an example, one sees that it agglutinates the series as follows :

	H agglutination.	O agglutination.
Strains agglutinated to the titre limit of Serum 23	16 strains .	5 strains.
Strains agglutinated to less than the titre limit	17 ,, .	24 ,,
Strains not agglutinated at all . . .	12 ,, .	16 ,,

To investigate how far such relationship extends it was necessary to carry out absorption tests, and these were therefore performed in the manner already described. Each serum was absorbed with the heterologous strain, and then tested against the homologous one for the presence or absence of H and O agglutinins. It was possible in several cases to carry out the mirror test of reciprocal absorption, and so establish the complete identity or not of the antigens concerned.

Without reproducing all the agglutination and absorption protocols here, it will suffice to say that of the 45 strains, 20 at once declared themselves as being serologically identical in respect of both H and O antigen. All 20 strains were biochemically typical and non-hæmolytic. In all cases in which reciprocal absorption was performed, it was complete, showing that no organism possessed any antigen not possessed by another—that is to say, the group was serologically entirely homogeneous.

Of these 20 strains, 9 were quite recently isolated ones, received by the kindness of Capt. Pasricha from the School of Tropical Medicine, Calcutta. They all proved to be biochemically typical, non-hæmolytic strains. Six gave good agglutination with H antibody. The remaining 3, though motile, did not, until concentrated flaggellar suspension had been prepared. This observation confirms similar ones on previous occasions, and demonstrates the fact that even a recently isolated cholera culture may be deficient in flagellar antigen and give a poor agglutination response.

It was found on more than one occasion that if a cholera strain is plated out and many colonies picked, a few of them are so poor in flagellar antigen as to give no, or hardly any, H agglutination until a concentrated flagellar suspension has been prepared. These results may explain the inagglutinability of certain cholera strains that are occasionally reported, for O titres of cholera sera are frequently negligible.

As regards the response of the recently isolated strains to the somatic antibody, it was found that, with the exception of 2 strains, it was a poor one; here, also, earlier observations on the inferior somatic agglutinability of most cholera strains are confirmed.

It would appear that the inferiority of the somatic agglutination is due to the poverty of the strains themselves in the somatic antigen, because it has been frequently observed that a serum may give a poor O titre with its own homologous strain, but a better one with heretologous strains.

Sera were prepared against 3 of these recently isolated strains, viz. 3639, 3640, 3641, in the hope of obtaining a better O serum than was achieved with the strains already employed, but the difficulty of obtaining good O serum

Classification of 45 Cholera and Cholera-like Vibrios.

Biochemically typical
Biochemically atypical, hæmolytic and non-hæmolytic
Non-hæmolytic
Hæmolytic
Serologically related to, but not identical with, *V. choleræ*. (3 strains: 722, 723, 3654.)
Not agglutinated by cholera serum. (7 strains: 559, 1548, 2084, 2902, 3651, 3652, 3653.)
Serologically related to, but not identical with *V. choleræ*; heterogeneous amongst themselves. (10 strains: 30, 685, 686, 718, 721, 2890, 3409, 3646, 3649, 3650.)
Not agglutinated by cholera serum. (2 strains: 3647, 3648.)
Serologically identical with *V. choleræ*; homogeneous amongst themselves. (20 strains: 17, 23, 27, 29, 1633, 2463, 2889, 2952, 3241, 3242, 3243, 3637, 3638, 3639, 3640, 3641, 3642, 3643, 3644, 3645.)
Serologically related to *V. choleræ*, but not identical; heterogeneous amongst themselves. (2 strains: 717, 3636.)
Not agglutinated by cholera serum. (1 strain: 689.)

TABLES I–III.—*Antigenic Analysis of Three Vibrio Strains. 23, a Typical Cholera Biochemically Atypical, Hæmolytic*

TABLE

Antigen.	Serum 23 unabsorbed. 1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	Absorbed with 2890. 1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.
23 H	++	++	++	++	++	++	++	++	++	+	–	–
23 O	++	++	++	+	+	–	–	–	–	–	–	–
2890 H	++	++	++	++	+	+	–	–	–	–	–	–
2890 O	++	++	++	+	–	–	–	–	–	–	–	–
1548 H	++	+	tr.	–	–	–	++	+	–	–	–	–
1548 O	–	–	–	–	–	–	–	–	–	–	–	–

TABLE

Antigen.	Serum 2890 unabsorbed. 1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	Absorbed with 2890. 1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.
23 H	++	++	++	++	++	+	–	–	–	–	–	–
23 O	++	++	+	+	–	–	–	–	–	–	–	–
2890 H	++	++	++	++	++	++	–	–	–	–	–	–
2890 O	++	++	+	+	–	–	–	–	–	–	–	–
1548 H	–	–	–	–	–	–	–	–	–	–	–	–
1548 O	–	–	–	–	–	–	–	–	–	–	–	–

TABLE

Antigen.	Serum 1548 unabsorbed. 1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	Absorbed with 2890. 1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.
23 H	++	+	tr.	–	–	–	++	+	–	–	–	–
23 O	–	–	–	–	–	–	–	–	–	–	–	–
2890 H	–	–	–	–	–	–	–	–	–	–	–	–
2890 O	–	–	–	–	–	–	–	–	–	–	–	–
1548 H	++	++	++	++	++	++	++	++	++	++	++	++
1548 O	++	++	+	+	+	–	++	++	+	+	+	–

was again demonstrated. In only 1 case was it successful, though, as before, there was no difficulty in the production of high H titres. Cross-agglutination and cross-absorption experiments were carried out as before, and all the nine strains were found to conform serologically with the *V. choleræ* group already established. A set of 20 cholera strains, all identical in their serology, was thus demonstrated.

The remaining 25 strains, whether biochemically typical or not, whether hæmolytic or not, were at most only serologically related to, but never identical with, this group of obviously typical cholera vibrios.

It is on the basis of these numerous cross-agglutinations and cross-absorptions that it has been possible to classify this series of 45 strains as follows:

Biochemically Typical Strains.

A. *Non-hæmolytic.*

(1) A group of 20 strains the members of which are all serologically identical as regards both flagellar and somatic antigens and all of which had been received labelled *V. choleræ*, and are to be regarded as typical cholera strains.

(2) Two strains which are related either in the flagellar or somatic antigens

Culture; 2890, a Biochemically Typical, Hæmolytic Strain Isolated at El Tor; 1548, a Strain Isolated in Alexandria.

I.

Absorbed with 1548.							Absorbed with 23.					
1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.		1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.
++	++	++	++	++	+	.	−	−	−	−	−	−
++	+	+	+	−	−	.	−	−	−	−	−	−
++	++	++	++	+	+	.	−	−	−	−	−	−
++	++	+	+	−	−	.	−	−	−	−	−	−
−	−	−	−	−	−	.	−	−	−	−	−	−
−	−	−	−	−	−	.	−	−	−	−	−	−

II.

Absorbed with 1548.							Absorbed with 23.					
1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.		1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.
++	++	++	++	++	+	.	−	−	−	−	−	−
++	++	+	+	−	−	.	−	−	−	−	−	−
++	++	++	++	++	++	.	++	++	+	+	−	−
++	++	+	+	−	−	.	++	+	+	−	−	−
−	−	−	−	−	−	.	−	−	−	−	−	−
−	−	−	−	−	−	.	−	−	−	−	−	−

III.

Absorbed with 1548.							Absorbed with 23.					
1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.		1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.
−	−	−	−	−	−	.	−	−	−	−	−	−
−	−	−	−	−	−	.	−	−	−	−	−	−
−	−	−	−	−	−	.	−	−	−	−	−	−
−	−	−	−	−	−	.	−	−	−	−	−	−
−	−	−	−	−	−	.	++	++	++	++	+	+
−	−	−	−	−	−	.	++	++	+	+	+	−

to *V. choleræ*, but are not identical; they were not capable of absorbing all anti-bodies from a cholera serum. These 2 strains were also related to, but not identical with, one another.

(3) One strain which was not agglutinated by cholera serum.

B. *Hæmolytic.*

(1) A group of 10 strains behaving antigenically like Group A2, *i.e.* their antigens are related to those of cholera, but not identical with them. The group is heterogeneous in itself.

(2) Two strains quite inagglutinable by cholera serum.

While this work was in the course of completion, Abdoush (1932) published the results of a similar serological study of *V. choleræ* and allied strains. In it he described a group of biochemically typical hæmolytic vibrios, which he found to be serologically identical with *V. choleræ*. He proposed restricting the name El Tor to these hæmolytic cholera strains. None of the 12 culturally similar strains, *i.e.* biochemically typical, but hæmolytic vibrios investigated here, showed the antigenic identity which he described.

Biochemically Atypical Strains.

A group of 10 strains, some of them hæmolytic, others non-hæmolytic. Three were related to, but not identical with, *V. choleræ*. Seven showed no serological relationship with this organism. They were all heterogeneous amongst themselves.

The following diagram gives a survey of this classification and the identity of the strains concerned.

In the following agglutination and absorption Tables I–III some idea is given of the serological relationship that exists between a typical cholera strain and 2 other cholera-like cultures.

The tables show the result of cross-absorption and agglutination, both flagellar and somatic ; for the former a concentrated flagellar suspension was used.

From these tables the following observations may be made:

RELATIONSHIP IN RESPECT OF FLAGELLAR ANTIGEN.

While the El Tor and Alexandria strains have H antigen in common with the true cholera strain 23, neither is capable of absorbing all the H antibody from the cholera serum, nor can the cholera strain exhaust their respective sera of H antibody.

The El Tor and Alexandria strains have no H antigen in common ; there is no cross-agglutination between them in respect of this antigen.

RELATIONSHIP IN RESPECT OF SOMATIC ANTIGEN.

Somatic cross-agglutination exists only between the cholera culture and the El Tor strain. While the El Tor strain depletes the cholera serum of all its somatic antibody, the converse does not occur. Therefore the El Tor strain must contain the somatic antigen of the cholera vibrio with an extra somatic factor in addition.

The Alexandria culture has no somatic relationship with either of the two other strains ; there is neither cross-absorption nor cross-agglutination between them. The antigenic content of these three strains may be expressed as follows :

Strain.	H-antigen factors.	O-antigen factors.
V. choleræ 23	A, B	I
El-Tor 2890	A, C	I, III
Alexandria 1548	B, D	II

DISCUSSION.

From the agglutination and absorption tests carried out with 45 strains of cholera and cholera-like vibrios may be deduced the fact that *V. choleræ* is a species of organisms serologically homogeneous, but related antigenically to a variety of cholera-like vibrios. This relationship may extend either to the

flagellar antigen or, contrary to the findings of Shousha (1931), to the somatic antigen.

Of the 25 cholera-like organisms investigated, 13 were related to *V. choleræ* in respect of H antigen and 9 in respect of O antigen. It follows, therefore, that the cholera vibrio may not be diagnosed by agglutination alone, even though that test be limited to the somatic factor. For complete diagnosis it is necessary to resort to absorption. With the application of this test the 20 strains of *V. choleræ* investigated here were seen to possess complete identity of antigen, and the cholera-like organisms could easily be differentiated.

Abdoush's (1932) report of hæmolytic strains which were otherwise indistinguishable both culturally and serologically from the typical *V. choleræ* could not be confirmed.

At the time of publication a paper by van Loghem (1932) has appeared, in which he states that he could not find any "noteworthy serological differences" between typical *V. choleræ* and the 6 hæmolytic El Tor strains with which he worked. However, of the 12 biochemically typical but hæmolytic strains examined here, 2 were serologically unrelated to *V. choleræ* and 10 were at once differentiated by the absorption test; they were serologically related to, but not identical with, *V. choleræ*.

The method employed by Balteanu of using an enriched flagellar suspension for agglutination purposes has proved valuable in the detection of affinities between organisms which would otherwise have been concealed.

The extent to which such affinities exist in respect of both flagellar and somatic antigen was worked out in detail for 3 related vibrios, one, a typical *V. choleræ*, and the others cholera-like organisms, isolated at El Tor and Alexandria respectively.

SUMMARY.

1. As other workers have already demonstrated, the cholera vibrios possess both flagellar and somatic antigen.

2. All true non-hæmolytic cholera vibrios possess the same flagellar and somatic antigen.

3. Cholera-like organisms may possess antigen in common with *V. choleræ*, and thus be serologically related to this organism. This relationship may refer to either the flagellar or somatic antigen.

4. To differentiate such strains from *V. choleræ*, the agglutination test is, therefore, insufficient; the absorption test is essential.

5. No hæmolytic strains in the series possessed antigen identical with that of *V. choleræ*.

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A SPECIFIC PRECIPITIN REACTION ASSOCIATED WITH THE GROWTH ON AGAR PLATES OF MENINGOCOCCUS, PNEUMOCOCCUS, AND *B. DYSENTERIÆ* (SHIGA).*

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Received for publication July 15th, 1932.

THE characteristic appearances which form the subject of this paper were observed during the course of some experiments in which attempts were made to produce "rough" variants from freshly isolated strains of meningococcus and from "smooth" strains of pneumococcus (Type I) by growing them in the corresponding immune-serum. The immune-serum cultures were plated on various kinds of agar medium with the hope of finding one that would clearly distinguish between the "R" and "S" types of colony.

SPECIFIC HALOES ROUND COLONIES OF MENINGOCOCCUS.

In the search for a differential medium for "R" and "S" strains of meningococcus two cultures were selected for comparison: "*C.M.*," a strain with Type I affinities, which was isolated from the nasopharynx of a "contact" early in the year 1926; and "*Ipswich* 2," a Type I strain from the outbreak of 1931. On November 17th, 1931, agar plates were prepared as follows: (1) With the addition of 2·5 per cent. sodium chloride; (2) with 5 per cent. normal horse-serum; (3) the same as (2), but with 2·5 per cent. sodium chloride; (4) with 5 per cent. anti-meningococcus serum (Type I); and (5) the same as (4), but with 2·5 per cent. sodium chloride. The salt was added with the idea that it might intensify the "rough" appearance of colonies that were composed of salt-sensitive cocci. The anti-meningococcus serum was a mixture from four horses which were being immunized with Type I cultures; the mean agglutination titre was 1 in 450. Agar plates prepared in this way were inoculated with each of the two strains.

Three days later a delicate halo was seen round the colonies of the recent strain on the agar to which anti-meningococcus serum had been added, but not round those of the old laboratory strain. The haloes were apparent on the plates, both with and without 2·5 per cent. sodium chloride.

These observations, which are supported by further work, indicate that the halo represents a specific precipitate which is formed by the interaction of the antibody in the serum with the specific polysaccharide of the meningococcus (*cf.* Rake, 1931); this is set free from the cocci in the colony by a process of secretion or autolysis, and diffuses into the agar round the colony. Similar

* A demonstration of this reaction was given by the author at a meeting of the Biochemical Society which was held on February 12th, 1932. Figs. 1, 2, 3 and 4 are photographs taken from the immune-serum agar plates that were then exhibited.

haloes were demonstrated soon afterwards by means of the same technique with virulent Type I pneumococcus and with an "S" strain of dysentery bacillus (Shiga). The method is doubtless applicable to many bacteria, provided that the requisite conditions for the production of the haloes are fulfilled.

The General Features of the Haloes round Meningococcal Colonies.

The halo forms a delicate annular opacity round the colony, and is best seen against a black background when the plate is illuminated by a concealed electric light; magnification by means of a reading lens is an advantage. For effective demonstration the colonies should be well separated from each other; the implantation of not more than 4 colonies on a plate measuring 9 cm. in diameter allows ample room for expansion of the haloes. A convenient method for securing adequate separation is to stab the agar with a needle-pointed platinum wire which has been charged with the culture from an agar slope. The density of the precipitate is so slight that the halo is not visible under the microscope (Fig. 1).

The haloes round Group I colonies remain fairly constant in size during the first three days, and then expand at a rate which is out of proportion to the growth of the colony, so that after about ten days they have coalesced and cover the whole surface of the agar in the plate. The observation has been made that before merging into each other, the opposing curved edges of adjacent haloes may become straight and run parallel. Table I summarizes observations on the comparative increase in size of the colonies and their haloes. The measurements are expressed as a ratio, the numerator of which gives the mean area of the colonies, and the denominator the mean annular area in square millimetres. In this calculation of the area of the haloes the assumption is made that the precipitate does not extend over the site of the colony. The correctness of this assumption cannot be verified by inspection, but it may be presumed that the concentration of the polysaccharide is greatest in the region of growth and autolysis, and it is known that a specific precipitate tends to dissolve when one of the reagents is present in excess. Actually, in a well-developed halo a clear narrow ring may often be observed near the edge of the colony. The question, however, is of little moment, because calculations which include the area of the colony give a proportional series of ratios. We may infer that the data indicate a gradual diffusion outwards of the polysaccharide into the agar.

Proof of the physico-chemical basis of the reaction and of the resulting halo will be found in Tables II and III; the data they contain make it evident that in these experiments the haloes increased in size while the colonies remained stationary. Moreover, the haloes expanded under conditions—at room temperature and in the cold room—which are incompatible with the survival of the meningococcus.

The Specificity of the Haloes.

The evidence indicates that the reaction which produces the haloes is both species-specific and type-specific, or at least group-specific. Thus, meningococcal colonies do not produce haloes when they are grown on agar containing

TABLE I.—*Comparative Rate of Increase of Meningococcal Colonies (Group I) and their Haloes during Growth of the Colonies for 10 Days at 37° C.*

	Day of observation.						
	1.	2.	3.	4.	6	8.	10.
Ratio = mean area of colony/mean area of halo in sq. mm.	72·9 / 137·6	17·4 / 37·6	38 / 75	44 / 206	74 / 487	88 / 963	88 / *
The above figures expressed as a simple ratio	1 / 1·9	1 / 2·2	1 / 1·9	1 / 4·7	1 / 6·5	1 / 10·9	. / ..
Number of colonies (and haloes) observed	9	29	9	20	20	12	12

* The haloes could not be measured because they had spread over the plate and coalesced.

TABLE II.—*Mean Measurement in Square Millimetres of Eight Meningococcal Colonies (Group I) and their Haloes. The Plates on which the Colonies were Implanted were kept for 6 Days at 37° C.; thereafter for 4 Days at Room Temperature; and then for 11 Days in the Cold Room. The Data show that the Haloes have Increased in Size at Temperatures below those which are Compatible with the Life of the Meningococcus.*

	Day of observation.								
	2.	4.	6.	8.	10.	12.	16.	21.	
	37° C. for 6 days.			Room temperature for 4 days.		Cold room for 11 days.			
Ratio = mean area of colony/mean area of halo in sq. mm.	18·1 / 36·5	36·6 / 210·4	64 / 455	68 / 671	70 / 861	70 / 862	69 / 1007	70 / 1108	
The above figures expressed as a simple ratio	1 / 2	1 / 5·7	1 / 7·1	1 / 9·9	1 / 12·3	1 / 12·3	1 / 14·6	1 / 15·7	

TABLE III.—*Rate of Increase of the Haloes Round a Colony of a Group I and of a Type III Meningococcus when kept successively at 37° C., in the Cold Room, and at Room Temperature.*

Strain.	Day of observation. 2. 2 days at room temperature.	4. 2 days in cold room.	9. 7 days in cold room.	15. 6 days at room temperature.	21. 12 days at room temperature.	28. 19 days at room temperature.	35. 26 days at room temperature.
No. 116, Group I . Ratio = mean area of colony / mean area of halo in sq. mm.	39 / 94	39 / 138	44 / 133	45 / 370	45 * / 570	45 / 972	45 / 1475
No. 114, Type III Ditto	19·6 / 58·9	19·6 / 75·4	24 / 230	24 / 230	24 * / 356	24 / 682	24 / 883

* The halo showed concentric rings.

TABLE IV.—*Presence or Absence of Haloes when Virulent ("S") Strains of Types I, II and III and a Non-virulent ("R") Strain of Type I Pneumococcus were Implanted on Agar Plates, which contained Anti-pneumococcus Serum obtained from Horses which were Immunized against the Various Types.*

Source and date of sample.	Serum used in plate. Types used for immunizing horse.	Approximate "Felton" units per c.c. T.₁S.	T.₂S.	Agglutination titre. T.₁S.	T.₁R.	T.₂S.	Type of colony implanted on plate. T.₁S.	T.₁R.	T.₂S.	T.₃S.
"Ena" 25.ii.32	I and II	1500	<50	1/320	..	1/60	(5 / ++118)	0	0	0
"Ena" 26.iv.32	,,	500	100	1/160	1/32	1/60	(7 / ++106 *)	0	0	0
"Alfonso" ,,	,,	750	<25 on 29.iii.32	1/160	1/32	1/60	(19·6 / ++58·9)	±	±	0
"Loys" ,,	I	100	..	1/120	1/16	..	0	0	0	0
"Radio" ,,	II	..	50	1/16	1/4	1/160	±	0	±	0
"Edwards" ,,	II	..	..	1/16	1/16	1/40	±	0	±	0
"Nippy" ,,	I "Rough".	..	..	..	1/4	..	(44·3 / +132·7 *)	(19·6 / +134·4 *)	0	0
,, 17.v.32	I "Rough".	..	..	1/2	1/8	..	(10 / ±9·6 *)	(10 / +9·6 *)	(7 / ±71)	?±

Note.—++ = typical well-developed halo; + = faint halo; ± = very faint and narrow halo; 0 = no halo.
The ratio in brackets represents the mean area of the colony the mean area of the corresponding halo in square millimetres.
T.₁S., T.₂S., T.₃S., T.₁R. = Type I "smooth," Type II "smooth," Type III "smooth," and Type I "rough."
* The halo showed concentric rings.

normal serum or anti-pneumococcus serum. With regard to the type-specificity of the reaction, the results that have been obtained are consistent with the antigenic overlapping which is generally believed to characterize the types of meningococcus as classified by Gordon and Murray. For example, Type III strains, when implanted on a Type I serum agar, may give haloes which are as well developed as those produced by Type I strains. In one experiment a Group II strain gave the reaction on a Group I serum agar, but agglutination tests with absorbed sera proved that the strain possessed a proportion of Type I antigen.

The Applicability of the Method for Distinguishing between "Rough" and "Smooth" Variants of Meningococcus.

A mixed anti-meningococcus serum (Group I) which was obtained a fortnight previously from six horses and had a mean agglutination titre of 1 in 900 was added to agar plates in a concentration of 5 per cent.; 46 strains of meningococcus were implanted on the plates.

Twenty-eight of the strains gave no halo. Eight of these were Type I strains, and 1 was a Type III strain; 19 of the number were certainly or probably members of Group II, and 6 of these were recent strains, which were definitely assignable to this group. All of the Type I strains were at least fifteen months old, and 5 of them were four or more years old.

Eighteen of the strains produced haloes. Of these, 14 had been received less than one year before; they were strains from the recent outbreak; and none was more than fifteen months old. The strains in this group comprised 6 of Type I, 10 of Type III, and 2 of Group I.

The ability of the old strains to produce type-specific polysaccharide is thus apparently much less than that of the recent strains; in this respect they have become degraded.

SPECIFIC HALOES ROUND COLONIES OF PNEUMOCOCCUS (TYPE I).

The following experiment was carried out on November 26th, 1931: An agar plate was prepared which contained 5 per cent. normal horse-serum and another with 5 per cent. of an anti-pneumococcus serum (Type I); this sample was derived from two horses, and its protective value in mice was less than 50 "Felton" units per c.cm. The following cultures were implanted on each plate by stab inoculation: (1) A strain of virulent Type I pneumococcus; (2) a strain of virulent Type II pneumococcus; (3) a "rough" strain of Type II pneumococcus; (4) a "smooth" strain of *B. dysenteriæ* (Shiga); and (5) "Ipswich 2," a recently isolated strain of Type I meningococcus. The plate containing the anti-pneumococcus serum showed haloes only round the colonies of the virulent Type I pneumococcus; the plate with the addition of the normal horse-serum showed no haloes.

This experiment was the first of its kind, and is representative of others that gave similar results. The reaction is apparently species-specific and type-specific. A number of observations on the type-specificity of the reaction are collected in Table IV.

The General Features of the Haloes.

The haloes produced by the pneumococcus are similar to those which have already been described for the meningococcus, but they show much less tendency than these to expand and spread over the plate—a difference which indicates that the pneumococcus is less easily autolysable than the meningococcus, or that its type-specific polysaccharide diffuses less readily through the agar than that of the meningococcus. The halo of the pneumococcus consists, as a rule, of a delicate annular area, which is so thin that it cannot be recognized under the microscope (Fig. 2). The precipitate may, however, be so dense that the colonies resemble the mucoid type which is occasionally seen in the coli-typhoid group of bacteria; the haloes are then easily recognized under a low power of the microscope (Fig. 3).

When the appropriate conditions are arranged, the specific precipitate is deposited as a band on each side of a streak of growth on an agar plate; and round the growth in a deep agar stab tube. These methods, however, offer no practical advantages as compared with the method of implanting colonies on an agar plate.

The Relation of Antibody Concentration and "Smoothness" of the Culture to the Production of Haloes.

In the first experiment, which has been described above, a series of agar plates were made, in addition to the one that received 5 per cent. of immune serum, and to them was added 0·5 per cent., 0·05 per cent., and 0·005 per cent. of the serum. Haloes appeared only on the plate which contained 5 per cent. of the serum. As already stated, this sample of serum was of weak potency in mouse-protection tests. An agar plate was therefore prepared with 5 per cent. of a comparatively strong serum—approximately 1000 "Felton" units per c.cm.—and another with 5 per cent. of this serum after it had been diluted ten-fold with normal saline. Both plates were spread with a virulent culture of a Type I pneumococcus. After suitable incubation the colonies on the pure serum plate resembled the mucoid colonies already referred to, whereas the haloes round those on the diluted serum plate were much more delicate.

Another important factor in the production of haloes is the state of "smoothness" or, in other words, virulence of the culture. Thus, if the cultural conditions are such as to be incompatible with full virulence, the haloes are less obvious, and indeed may fail to develop, although a test shows that the culture possesses a fair degree of virulence for mice. In one experiment three colonies, which had the microscopical features of virulent colonies, were picked off into broth; two of these were surrounded by a halo and the third had none. The broth cultures of the two haloed colonies killed mice in a dose of 0·000001 c.cm.—the limit of the test; the culture of the colony without a halo killed in a dose of 0·0001 c.cm., but not 0·00001 c.cm. Cultures that have been incubated for a week or longer lose their power of forming haloes when implanted without fresh subcultivation.

The Applicability of the Method for Distinguishing between "R" and "S" Variants of Pneumococcus.

In the course of this work many experiments were carried out on the production of "R" colonies by growing an "S" culture in the homologous immune-serum, and it was found that the transformation was by no means easily effected. The concentration of antibody seems to be an important factor in inducing the change, but subcultivation in plain broth is equally effective. The detection of "R" variants in work of this nature is facilitated by the use of an immune-serum agar plate. Haloed colonies can certainly be regarded as virulent, but, on the other hand, as has already been indicated, the absence of haloes may be due to the scarcity of "S" cocci and the predominance of "R" cocci in the colony; or conceivably to a partial degradation of the cocci, the virulence of which may again become exalted when they are injected into mice.

The Formation of Haloes Round "R" Colonies of Pneumococcus.

When a "rough" Type I pneumococcus is sown upon a "smooth" Type I immune-serum plate a faint and narrow halo may appear, after a considerable period of incubation at 37° C., or even after storage at room temperature following incubation at 37° C. Haloes of this kind may be so delicate that close scrutiny under suitable lighting conditions is necessary to reveal them. These haloes are presumably brought about by the interaction of the common "R" polysaccharide or the somatic nucleoprotein with the corresponding "R" antibody in the type-specific serum, when this is added to the agar plate.

A horse was immunized with an "R" Type I pneumococcus. An agar plate containing a sample of the serum after a course of immunization

DESCRIPTION OF PLATE.

FIG. 1.—(× 1). A group of colonies of a recently isolated strain of Type I meningococcus with haloes, and a group of colonies of an old laboratory culture (Type I) without haloes. The colonies were implanted on a plate to which a Type I antimeningococcus serum had been added.

FIG. 2.—(× 1). Five colonies of a virulent Type I pneumococcus with haloes. The colonies enclosed within the squares were inoculated from a "rough" variant of the virulent strain. The agar contained a Type I anti-pneumococcus serum.

FIG. 3.—(× 3). Illustrating unusually dense haloes round colonies of a virulent strain of Type I pneumococcus.

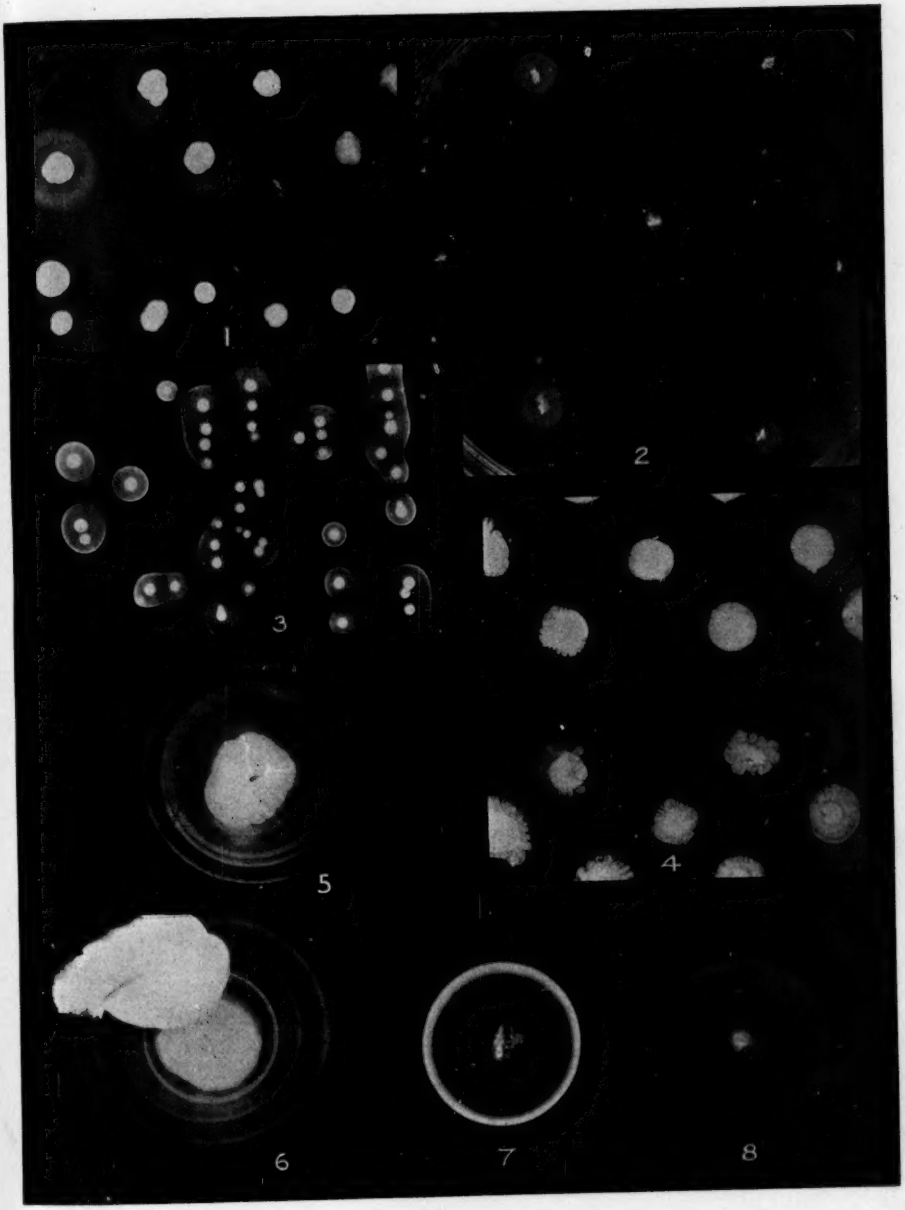
FIG. 4.—(× 1). A drawing from a photograph of "smooth" colonies of *B. dysenteriæ* (Shiga) with haloes, and colonies of the "rough" variant of the same strain with no haloes.

FIG. 5.—(× 2). Liesegang rings in the halo surrounding a colony of Type III meningococcus. A Group I immune-serum had been added to the agar.

FIG. 6.—(× 2). Liesegang rings in the halo surrounding a colony of Group II meningococcus, which, however, contained a proportion of Type I antigen. The concentric formation is evident in spite of the intrusion of a contaminating colony. A Group I immune-serum had been added to the agar.

FIG. 7.—(× 3). Liesegang rings in the halo surrounding a colony of a virulent Type I pneumococcus; the culture was implanted on agar containing anti-pneumococcus serum (Type I).

FIG. 8.—(× 1). Liesegang rings obtained by depositing a drop of 1/1000 dilution of the specific polysaccharide of *B. dysenteriæ* (Shiga) upon a rabbit immune-serum agar plate.



Petrie.

was sown with various types of pneumococcus. Definite haloes were seen round the Type I " rough " and the Type I " smooth " strains; although faint, they were of the usual size for pneumococcus; a very faint and narrow halo was also seen round a Type II " smooth " colony (see Table IV).

We may expect that an " R " culture will produce haloes of the " rough " variety in the presence of an anti-" R " serum or of any of the type-specific sera; and that any type of " S " pneumococcus will produce haloes of the " rough " variety in the presence of an anti-" R " serum.

THE PRODUCTION OF HALOES IN AGAR TO WHICH ANTI-PNEUMOCOCCUS SERUM (TYPE II) HAS BEEN ADDED.

The serum from two horses which were being immunized against Type II pneumococcus, but which had not reached a high titre of potency, as judged by protection tests in mice, was added to agar plates, and observations were made on colonies of various types of pneumococcus that had been implanted into the immune-serum agar. Haloes appeared round both Type II " S " and Type I " S " colonies, but they were extremely tenuous and difficult of observation. If we may judge from their appearance, it is probable that these haloes were such as are associated with the " R " type, and that they were not, therefore, type-specific.

SPECIFIC HALOES ROUND COLONIES OF *B. DYSENTERIÆ* (SHIGA).

Haloes have been seen round " smooth " colonies of *B. dysenteriæ* (Shiga), when 5 per cent. of anti-dysentery serum (Shiga) from the horse had been added to the agar; the " rough " strain of the same culture gives no haloes (see Fig. 4). It will be noted that one set of the colonies reproduced in the figure present the characters of the " rough " variant, and the other set those of the " smooth " variant.

ATTEMPTS TO PRODUCE HALOES WITH OTHER PATHOGENIC BACTERIA.

Experiments have been made with *B. pestis*, *B. typhosus*, the gonococcus and *Staphylococcus pyogenes aureus*, but they gave negative results. The lack of success is devoid of significance, for the reason that exact information on the mode of preparation of the corresponding immune-sera is not available.

THE FORMATION OF CONCENTRIC RINGS IN THE HALOES PRODUCED BY THE MENINGOCOCCUS AND THE PNEUMOCOCCUS.

An interesting feature of the haloes that are produced by the meningococcus and the pneumococcus is the appearance, under conditions that have not as yet been precisely defined, of concentric rings, which are reminiscent of the Liesegang rings.

Concentric Rings Round Meningococcus Colonies.

On April 29th, 1932, a pooled serum was prepared from the bleedings, taken on April 22nd, 1932, of six horses, which were being immunized against

Group I strains of meningococcus ; the mean agglutination titre of the mixture was 1 in 600. Five per cent. of this serum was added to an agar plate, which was then implanted by stab inoculation with 4 cultures of meningococcus : (1) A Group I strain ; (2) an old laboratory Type I strain ; (3) a Type III strain ; and (4) a Group II strain which contained, however, some Type I antigen. The plate was incubated for five days at 37° C., and was then kept at room temperature for 19 days. The haloes of all the colonies were now found to show concentric rings ; the exact time of their appearance cannot be stated, but it is known that they originated while being stored at room temperature (Figs. 5 and 6). The appearances were similar in all, except that the rings round the colony of the old laboratory strain were considerably fainter than those round the other colonies ; there were five opalescent rings alternating with clear rings round each colony, and the zone immediately adjacent to the colony was clear ; the diameter of the concentric area, when measured across the centre of the colony, was 13–15 mm. The outer part of the original halo of each colony, that is, the portion beyond the outermost opalescent ring, had become ill-defined, and could not therefore be measured. The rings from within outwards appeared to be set at an increasing depth in the agar. Here it may be mentioned that, owing to the delicate structure of these haloes, whatever may be the bacterial species from which they arise, it has proved to be difficult, even by making a section of the colony and halo, to determine their position in the agar.

The next observation concerns two strains of meningococcus, No. 116 (Group I) and No. 114 (Type III). The haloes round the colonies of these strains were measured at intervals when the plates were kept for varying times at different temperatures (see Table III). Immune-serum agar plates were prepared on May 23rd, 1932 ; the serum was derived from two horses, and had an agglutination titre of 1 in 750. After 2 days' incubation at 37° C., 9 days' storage in the cold room, and 18 days at room temperature, faint rings were present in the haloes of both strains. The concentric arrangement was perceptible with difficulty and required careful observation. The rings round colony " 114 " were still present after 26 days at room temperature. The descriptive note then made was as follows : (1) A clear zone next colony ; (2) a faint ring ; (3) clear zone ; (4) slight hazy ring ; (5) slight clearing ; (6) rather thick ring ; (7) clear zone ; (8) thicker ring ; and (9) haze merging into the surrounding agar. The concentric area round the colonies was 14 and 16 mm. in diameter, when measured through the centre of the colonies.

Concentric Rings Round " Smooth " and " Rough " Colonies of Pneumococcus.

On February 2nd, 1932, an agar plate containing 5 per cent. of an anti-pneumococcus serum (Type I) with a protective titre of about 1000 " Felton " units was inoculated by means of a stab wire from 5 virulent Type I pneumococcus colonies and from 2 " rough " Type I colonies. Forty hours later the 5 " S " colonies showed a halo but not the " R " colonies. After some days' incubation at 37° C. the plate was kept at room temperature till February 12th, 1932, when a very faint haziness was seen in the neighbourhood of the " R " colonies ; the appearance contrasted with the readily seen haloes round the " S " colonies. The plate was maintained at room temperature until February 25th, 1932—that is, 23 days from the time of implantation—when rings were

seen round all the colonies on the plate. The " S " colonies showed a dense whitish ring at the circumference of the haloes ; this ring appeared to be whiter and thicker than when last observed 13 days before, and in addition the haloes showed concentric rings between the outer ring and the colony. There were 4 dense rings in all, and the zone immediately surrounding the colony was clear. The alternating dense and clear rings were of equal breadth. When a typical colony and halo were photographed ($\times$ 3), an extremely delicate and narrow ring was just visible on the photograph some distance beyond the outermost dense zone ; the diameter of this ring was 14 mm. (Figs. 2 and 7).

The haloes of the two " R " colonies also developed concentric rings, but these were much more delicate than those of the " S " colonies ; they required good illumination to see them properly. One of them showed three opalescent rings alternating with clear zones. The diameter of the halo, including that of the colony, was 8 mm.

Concentric rings were also noticed on some of the plates in the experiment of which the results are condensed in Table IV. Attention may be specially directed to the results of growing various " S " pneumococcal types and a Type I " rough " strain on an agar plate containing an anti-" R " serum from the horse to whose immunization reference has already been made. Samples of serum that were taken from this horse before the immunizing course was begun gave no haloes. A sample which was taken on April 26th, 1932, after 11 intravenous doses of suspensions of " rough " cocci, gave very faint haloes with a Type I " S " and a Type I " R " culture, but not with a Type II and a Type III culture. The rings were noticed on the plate after 5 days' incubation at 37° C. and 5 days at room temperature. A sample of serum which was taken on May 17th, 1932, after further immunization, gave definite haloes with Type I " S " and Type I " R " after incubation for 2 days at 37° C. and 4 days at room temperature ; the halo round the " R " colony was more distinct than that round the " S " colony. Very faint concentric rings were observed 2 days later. A week later a very faint halo was noted round the Type II " S " colony, and after 3 more days there was a suggestion of a partial halo extending only one-quarter of the way round the Type III " S " colony. The Type I " R " and " S " haloes contained 4 opalescent concentric rings alternating with clear zones ; the diameter of the outermost ring was 10–11 mm.

The Production of Haloes and Concentric Rings by Means of a Purified Bacterial Polysaccharide.

Through the kindness of my colleague, Dr. W. T. J. Morgan, an opportunity was afforded of testing whether a halo could be produced by adding to an immune-serum plate a small amount of the highly purified preparation of specific polysaccharide which he has isolated from Shiga's dysentery bacillus (Morgan, 1931).

A depression was made in the centre of an immune-serum agar plate, and into this there was placed a drop of a sterile syrupy solution of the " smooth " polysaccharide. Two kinds of immune-serum were available, one from a horse, and the other prepared by Dr. Morgan by immunizing a rabbit with a " smooth " strain of Shiga's dysentery bacillus. Next day the agar plate which contained the rabbit immune-serum showed an area of opalescence

in the form of concentric zones round the site of deposition of the polysaccharide, whereas the plate with the horse immune-serum showed only a slight opacity. After a week had elapsed the original halo on the rabbit immune-serum plate was surrounded by a uniformly opalescent circular area with a total diameter of 52 mm.

This experiment was supplemented by implanting agar plates containing each kind of immune-serum with the " R " and " S " variants of a strain of Shiga's bacillus. The " S " variant gave a halo on the horse-serum plate, but not on the rabbit-serum plate ; the " R " variant gave no halo on either plate.

The next experiment of this kind proved to be even more convincing. A 1/5 dilution of an anti-dysentery (Shiga) serum obtained from the rabbit by means of a " smooth " strain was added to agar plates in a concentration of 5 per cent.; the pure serum had an agglutination titre of 1 in 4000. Dilutions of 1/100, 1/1000, 1/10,000 and 1/100,000 were prepared from the specific polysaccharide, and to each of the dilutions a small fragment of agar was added ; the mixtures were then sterilized at a temperature of 130° C. A central depression, 5 mm. in diameter, was made in the agar, and a drop of each dilution was placed in the depression ; the admixed agar caused the drop to " set " quickly. The plates were incubated at 37° C. for 24 hours.

Ten minutes after the drop of 1/100 dilution was placed on the plate a circular area of uniform haziness with a diameter of about 15 mm. appeared. On the following day this plate showed a central opacity of 5 mm. diameter ; next, a less opaque zone of 8 mm. diameter ; and then a very faint uniform halo of 21 mm. diameter ; three days later the outer very faint halo had disappeared.

The plate on which the drop of the 1/1000 dilution was placed presented the appearance of a typical delicate halo. After only 2 hours there was a definite halo round the drop of the polysaccharide solution. Next day the central opacity had a diameter of 10 mm. ; this was surrounded by a clear ring 2 mm. in width ; and this again was bounded by an outer annular opacity with a total diameter of 22 mm. After 24 hours at room temperature the total diameter of the outermost edge of the halo was 28 mm. ; 2 days later the corresponding measurements from within outwards were 12, 4 and 32 mm. The appearance, as a whole, with its concentric zones and delicate annular opacity, simulated perfectly the haloes round bacterial colonies that have already been described (Fig 8).

The 1/10,000 dilution gave rise to the following appearances : a clear centre, an opaque ring, a clear ring, and an outer halo the diameter of which was 17 mm. The 1/100,000 dilution presented a similar picture.

In addition to the rabbit serum the serum from two immune horses, " Bedford " and " Standard," was tested in the same way with the same dilution of polysaccharide ; the agglutination titre of the first was 1/10,000 and of the second 1/125.

The resulting appearances were very similar to those on the rabbit serum-agar plate, but the opacities were denser ; of the three sera the serum of " Bedford " gave the most opaque haloes.

It is significant that for each of the three sera the 1/100 dilution was the least characteristic and the least opalescent. The concentration of the

polysaccharide was probably so much in excess of the amount of antibody present as to inhibit the formation of the precipitate.

On the fifth day of observation the 1/10,000 precipitate on the rabbit immune-serum agar was denser than that of the 1/1000 drop ; and the 1/100,000 precipitate was larger and denser than that of the 1/100 drop. The order of diminishing opacity of the various reactions on the plates which contained the serum from the two horses was : 1/10,000, 1/100,000, 1/1000 and 1/100. These results indicate that the appearances are determined by the relative concentration of the reacting substances, and that the densest precipitate corresponds to the optimal proportion.

The observations, as a whole, provide strong evidence that the haloes produced by *B. dysenteriæ* (Shiga), and doubtless those of the meningococcus and pneumococcus, are really, as has been surmised, formed by a precipitate which results from the interaction of the specific polysaccharide with the antibody in the immune-serum.

The Nature of the Concentric Rings in the Haloes Produced by the Meningococcus and the Pneumococcus.

There is every reason to believe that the concentric rings which have been described belong to the class of periodic structures that are known as Liesegang rings. From this point of view a special interest attaches to them, because the interacting substances which produce them are of a colloidal nature, whereas most of the work on this subject has been carried out with simple inorganic salts. A useful compendium of knowledge relating to the Liesegang phenomenon, with a discussion of the conflicting theories which have been advanced in explanation of it, will be found in the recent publication by Hedges (1932). After thirty-five years of research the problem of the mode of formation of these structures is still unsolved.

Bechhold (1905) carried out experiments with the object of reproducing ring structures with pure colloids. He added an immune-serum prepared in the rabbit by inoculating it with goat-serum to an equal volume of 1 per cent. gelatine, cooled the mixture to a gel in the ice-chest, and poured goat-serum above the column. After 24 hours in the ice-chest the goat-serum had diffused into the gelatine, with the result that two sharply separated cloudy rings of precipitate formed in the gelatine. Similar observations are reported by Reiner and Kopp (1927).

CERTAIN FACTORS THAT MAY INFLUENCE THE PRODUCTION OF SPECIFIC HALOES.

The Addition of Sugars and Polysaccharides to the Medium.

It is natural to think that the addition to the immune-serum agar of a particular sugar or polysaccharide may favour the formation of the halo by increasing the metabolism of the cell with respect to the production and the output of the specific bacterial polysaccharide. In order to test this possibility the following substances were selected for trial : (*a*) simple sugars—dextrose, galactose, mannose, and lævulose (0·5 per cent.) ; (*b*) polysaccharides—glycogen (0·5 per cent.), inulin, dextrin, and gum acacia (1 per cent.), and

soluble starch (0·1 per cent.) A virulent Type I pneumococcus and a recent strain ("Ipswich 2") of meningococcus were used for inoculating the immune-serum agar plates.

A preliminary experiment showed that for both organisms haloes were produced when one of the simple sugars mentioned was added to the medium, but that no haloes were formed in the medium when one of the polysaccharides (glycogen, inulin, etc.) was incorporated into it. A reasonable explanation seems to be that the relatively high concentration of a non-specific polysaccharide—a colloid with a high molecular weight—in a complex colloidal system had interfered with the formation of the specific polysaccharide precipitate. Morgan (1932) has shown that the precipitin reaction, as indicated by the production of anaphylaxis in the guinea-pig by means of the specific polysaccharide he has isolated from Shiga's bacillus, is not inhibited by the presence of a large excess of various non-specific polysaccharides. The physical conditions in his experiments and in the experiments under discussion are, however, so different that it is unsafe to draw a comparison between them. There has been no opportunity of carrying the inquiry further.

The Comparative Suitability of Horse Immune-serum and of Rabbit Immune-serum for the Production of Haloes.

Without entering into details it may be stated that the evidence we have obtained with pneumococcus (Type I) and *B. dysenteriæ* (Shiga) indicates that immune-serum from the rabbit is not so suitable for halo production as immune-serum from the horse. The explanation for this difference is not readily apparent. There is evidence that the percentage distribution of the globulin fraction in rabbit-serum is a good deal smaller than that in horse-serum (Robertson, 1912); this circumstance may conceivably influence the deposition of the insoluble protein in the precipitate. The delicacy of the haloes that were formed when the specific polysaccharide from Shiga's bacillus was deposited on a rabbit immune-serum plate as compared with those which appeared on a horse immune-serum agar plate constitutes evidence that is in agreement with this view.

There is another consideration that may be relevant in seeking for an explanation of the difference. Observations have been recorded by some workers which indicate that rabbit immune-serum and horse immune-serum are not mutually replaceable in anaphylaxis and complement-fixation experiments (Avery and Tillett, 1929; Morgan, 1932; and Lancefield and Vollmond). It may be that the difference in halo production is referable to this class of discrepant result, although, contrary to the experience of the authors cited, in the present work the horse immune-serum is more effective than that from the rabbit. The colloidal system *in vivo* or *in vitro* in which the reaction takes place is perhaps an important factor in determining whether precipitation will occur with a particular serum or not.

THE FORMATION OF NON-SPECIFIC HALOES ROUND BACTERIAL COLONIES.

During the course of this work pseudo-haloes were observed on one occasion round the deep lenticular colonies of a virulent Type I strain of pneumococcus

in a serum-agar plate. The agar contained 5 per cent. of a 25 per cent. solution of normal horse-serum in distilled water; the serum-water had been heated to 130° C. for 15 minutes. The haloes were not unlike the specific haloes, but were thinner and had a somewhat wavy margin; there was no clear area immediately round the colony, such as is often seen within the specific haloes. A control plate without the serum water showed no haloes. It may be supposed that the proteins in the serum which was added to the agar had been altered in some way by the heat employed during sterilization and thus rendered prone to precipitation.

Atkin (1922) described haloes round colonies of meningococcus, irrespective of type, on trypsin-broth-pea-agar plates, and suggested that they resulted from the precipitation of a salt of legumin by the alkali formed during the growth of the colony.

DISCUSSION.

The technique for producing specific haloes round bacterial colonies furnishes a method of observing and comparing the output of the specific polysaccharide during the active growth and autolysis of bacterial colonies. The method may prove to be useful for studying the function of the bacterial cell that relates to the specific polysaccharides. Further, it seems that the method provides a rational criterion for distinguishing between "rough" and "smooth" colonies. The attribute of "roughness" or "smoothness" is a secondary characteristic, which depends upon the presence or absence of the specific polysaccharide in the colony; moreover, the microscopical appearance of the colonies of "R" and "S" variants creates at times a false impression of the character of the bacteria of which they are composed.

While the observations which have been recorded in this paper were being prepared for publication a preliminary communication by Sia and Chung (1932) appeared, in which they describe annular opacities round colonies of pneumococcus. They employed the method for identifying types of pneumococcus in the course of work which had for its object the transformation *in vitro* of one type into another.

SUMMARY AND CONCLUSIONS.

1. Characteristic haloes have been observed round colonies of "smooth" strains of meningococcus, pneumococcus and *B. dysenteriæ* (Shiga), when these bacteria were grown on agar plates which contained the homologous immune-serum.

2. The haloes consist of a precipitate which is formed by the interaction of the specific polysaccharide with the homologous antibody.

3. A "rough" strain of pneumococcus, by virtue of the "rough" polysaccharide or the somatic nucleoprotein, forms haloes in the presence of a "rough" or a type-specific serum; the haloes round "rough" colonies are extremely tenuous.

4. The haloes that are produced by the meningococcus and by both "smooth" and "rough" variants of the pneumococcus represent in the initial stage a uniform deposit of the precipitate; at a later stage the precipitate tends to assume the form of Liesegang rings.

5. When the specific polysaccharide which has been isolated by Morgan from *B. dysenteriæ* (Shiga) is deposited in suitable concentration upon an immune-serum agar plate, the resulting precipitate takes the form of a typical halo with concentric zones.

6. The ability to form specific haloes constitutes a test for distinguishing between the "rough" and "smooth" variants of bacteria.

My best thanks are due to my colleague, Dr. Douglas McClean, for re-determining the type of the old laboratory strains of meningococcus, to which reference is made in the text; and to Dr. W. M. Scott (Ministry of Health Laboratory) for supplying recently isolated and typed strains of meningococcus.

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QUANTITATIVE ASPECTS OF IMMUNITY REACTIONS: THE COMBINATION OF ANTIBODIES WITH SIMPLE HAPTENES.

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Received for publication July 15th, 1932.

ONE of the most important lights on the mechanism of immunity reactions is that given by the work of Landsteiner (1920). In this he showed that precipitin formation by a protein, coupled by an azo-link with a specific group such as p-amino-benzene-arsonic acid (atoxyl) and the serum of a rabbit immunized with protein containing this specific group, is inhibited by the presence of excess of simple substances containing the specific group (in this case atoxyl). This suggested that such precipitin reactions take place in

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two stages—(1) combination of antibody with the specific group of the antigen, (2) some intramolecular change leading to precipitation ; and that this second step only takes place when the specific group is attached to a large molecule.

In pursuance of this idea we have sought to demonstrate the combination of antibodies with simple azo-dyes (which for convenience we will call haptenes) containing the specific group, and published a preliminary note on our work at the end of last year (Marrack and Smith, 1931).

Since then Landsteiner and Van den Scheer (1932) have shown that some such simple azo-dyes will actually form precipitates with the homologous antibody. This does not conflict with the above two-stage theory, and is of great importance in showing that, in these precipitin reactions, on the one hand the specific group is necessary, and on the other hand that a complex molecule such as a protein is not essential in order that a precipitate may be formed. It does not therefore affect the object of our work, which was to demonstrate directly the specific combination even when no precipitate was formed, and to get a relatively accurate measure of the amount and character of the combination.

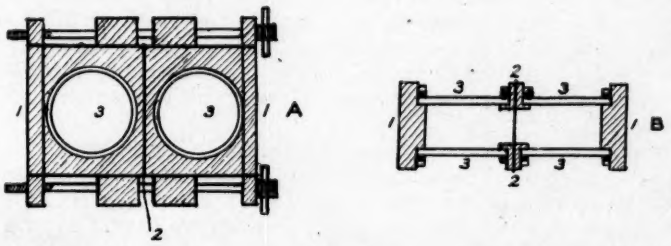

FIG. 1.—Apparatus for dialysis. A = front elevation ; B = cross section ; 1 = gilt brass ; 2 = collodion membrane ; 3 = silica disc.

METHODS.

The only methods available for the direct measurement of the amount of free and bound haptene in a haptene antibody mixture are based on dialysis, since haptenes will pass through a collodion membrane and antibodies will not. In using a dialysis method it is essential that the antibody-free fluid should be in equilibrium with that containing antibody. For example, if ultra-filtration were used, the water would pass through the membrane faster than the haptene, and concentration of free haptene would be less in the filtrate than in the filtered fluid (see Grollman, 1926), and with increasing concentration of the filtered fluid the equilibrium between haptene and antibody might be disturbed.

In order to obtain accurate results with the small amounts of fluid at our disposal we have used the apparatus shown diagrammatically in Fig. 1. The two halves were cut out of solid brass and heavily gilt. The cavities in the halves were exactly equal—about 2·3 c.c. The windows were closed with quartz discs fixed in by brass rings, which were screwed down on rubber rings. A collodion membrane was placed between the two halves, which were then clamped together. Thus two chambers were obtained walled with gilt brass

and silica and separated by a collodion membrane. Small quartz or hard glass beads were put in the chambers for stirring. The whole apparatus could be mounted on the periphery of a wheel 6 in. in diameter which rotated about eight times a minute. During dialysis the whole was kept in a Frigidaire refrigerator at a temperature of approximately 3° C.

The concentration of haptene in H could be measured spectrometrically through the quartz windows without removal from the dialyser, and thus the course of the dialysis could be followed and the attainment of a steady state insured. Tyrosin-azo-p-benzene-arsonic acid was measured at the wave-length 3100 Å, phenyl-azo-p-benzene arsonic acid and phenyl-azo-p-benzene-sulphonic acid at 3600 Å. It was possible to estimate the last two dyes satisfactorily with a micro-colorimeter, and this method was used instead of spectrometry in experiments towards the end of the work.

The collodion membranes were well washed by sucking through them first water and then haptene solution, and were kept soaking in haptene solution until used. Thus any error due to adsorption of haptene by the membrane was avoided.

It was found in preliminary work that considerable precautions had to be taken to get satisfactory results owing largely to the great dilution of the haptene solutions used, and consequent large percentage effect of small changes. The colour and ultra-violet absorption of the haptenes changed rapidly in the presence of soft glass. All solutions were made up with water distilled through hard glass.

The membranes were first tested for impermeability to protein, and then it was found how long it took for equilibrium to be reached when a solution of haptene was placed in one compartment and a buffer solution in the other. With tyrosin-azo-p-benzene-arsonic acid this took two days, and with phenyl-azo-p-benzene-arsonic acid one day. In the actual experiments a solution containing a known concentration of haptene was placed in one compartment (H), and in the other compartment (A) a solution containing both a known concentration of haptene and the protein solution to be tested. Dialysis was continued for the period found necessary for equilibrium to be attained, and in several cases for a day longer. The concentration of haptene in H was then measured. Since the original concentrations on both sides were known, the concentration in A could be calculated.

The concentration of free haptene in A is not exactly equal to the concentration in H owing to the Donnan effect, since the haptenes are anions, and the volume effect of the protein in A. Marrack and Hewitt (1927) have shown that the full theoretical Donnan effect for ideal solutions is not found with anions and serum proteins, and with the low concentrations of protein used here the effect is probably within experimental error. The concentrations of haptene should properly be calculated per kilo of water and not per litre of solution, but the estimations actually are made by volume This makes a difference of 1 to 2·5 per cent. and has been allowed for in calculating (F) the free haptene in A given in the tables. The bound haptene (B) is found by subtracting F from the total haptene in A.

The haptene mainly used in the early experiments was tyrosin-azo-p-benzene-arsonic acid. This was made as described by Pauli (1915); it was

twice recrystallized and dried over sulphuric acid. The amount of arsenic in all the preparations was above that calculated; in the sample used in the following experiments it was 117 per cent. of that calculated. For this reason, and because it proved difficult to prepare membranes readily permeable to this haptene, phenyl-azo-p-benzene-arsonic acid was used instead. This was prepared in the same way, twice recrystallized and dried. It contained the theoretical amount of arsenic, and diffused readily through our collodion membranes.

Phenyl azo-p-sulphonic acid was prepared by coupling phenol with diazo-benzene-sulphonic acid, and was twice recrystallized and dried.

The haptenes were brought into neutral M/100 solution; further dilutions for all purposes were made by dilution with M/15 phosphate buffer of pH 7·6 and were kept in Pyrex containers.

Antigens for immunization were prepared by coupling p-amino-benzene-arsonic acid and p-amino-benzene-sulphonic acid with horse-serum and test antigens by coupling with human serum. All were purified by repeated precipitation with acid and with alcohol. Rabbits were immunized by intravenous injections of 1 or 2 c.c. of approximately 2 per cent. solution twice a week.

In order to remove some of the non-specific proteins, globulin solutions were prepared from antisera and control sera by precipitation by 42 per cent. saturation with ammonium sulphate, dissolving the precipitate and dialyzing against 0·2 per cent. NaCl. The fluid was then centrifuged, the deposit rejected and the supernatant neutralized and NaCl added to 1 per cent. Before use these globulin solutions were dialyzed against the phosphate buffer solutions. Thus all solutions were used at pH 7·6, and the buffer maintained this pH during dialysis.

The globulin solutions from antisera were titrated by the method of optimal proportions (Dean and Webb, 1926). The arsenic equivalent in the tables is the millemolar arsenic (contained in the test antigen), equivalent of the antibody solution at optimal proportion.

Similar globulin solutions were also prepared from the sera of rabbits which had not been immunized with any protein derivatives. Most of these were animals which had been used for investigations on glandular fever. These solutions were used as controls and are called "normal." Besides these controls, solutions were made by precipitating the antibodies from specific sera by an amount of antigen slightly in excess of the optimal proportion. The antigen so used was dialyzed against phosphate buffer and the supernatant after precipitation dialyzed again. With these supernatant fluids it was possible to test the haptene-binding power of the non-specific proteins of antisera after the antibody had been removed.

The measurement of all small quantities was checked by weighing.

RESULTS.

We have to deal with two systems: (1) The sera from rabbits immunized with azo-proteins prepared from p-amino-benzene-arsonic acid, and the corresponding haptenes tyrosin- and phenyl-azo-benzene-arsonic acids; and

(2) the sera from rabbits immunized with azo-proteins prepared from p-amino-benzene-sulphonic acid, and the corresponding haptene, phenyl-p-amino-benzene-sulphonic acid. It was expected that the globulin of the first sera of the first system would combine specifically with the haptenes of this system and not those of the second system, and *vice versâ*.

Phenyl-azo-p-benzene-arsonic Acid (Table I).

Controls.—The amount of this haptene bound by non-specific rabbit serum globulin (Experiments 1 to 7) was not large, and in the lower concentrations it was approximately proportional to the concentration of globulin, and to the concentration of free haptene as shown by the constancy of the ratio $B/F \times P$. The serum globulin of rabbit 726, which had been immunized with azo-proteins prepared with p-amino-benzene-sulphonic acid, did not bind more than did the " normal " globulins. Nor did the globulin of the p-benzene-arsonic acid serum 743 from which the antibody had been precipitated as described under " Methods." When a much more concentrated solution of haptene was used as in the last experiment of this series (serum 742, antibody precipitated), the amount bound was not increased in proportion ; the limit of adsorption appears to have been reached.

Antisera.—The amount of haptene bound by the globulin of the specific antisera (Experiments 8 to 22) was from two to five times as high as that bound by control globulins. In three of these experiments, 10, 12 and 14, instead of putting equal haptene concentrations in H and A, the experiment was started with a lower concentration in H and a higher concentration in A than had been found in the previous experiments, 9, 11 and 13 at the end of dialysis. In 10, 12 and 14 a shift of haptene from A into H occurred—that is, the equilibrium distribution found in 9, 11 and 13 was approached, but it was never quite reached, the best agreement occurring in Experiments 9 and 10. The fact that the same distribution was approached from either side shows that there is a definite equilibrium point. In experiment 20 the amount of haptene bound appears to have reached the maximum for this solution—about seven times the maximum bound by the control in Experiment 7.

Tyrosin-azo-p-benzene-arsonic Acid (Table II).

Controls.—This haptene was bound by the control globulin to about the same degree as was phenyl-azo-p-benzene-arsonic acid. The proportion between amount bound and protein concentration was not so good. In Experiment 25 the dialysis was started with a low concentration in H and high in A, as in Experiment 34 below, but the equilibrium distribution attained was the same as in the other experiments with control globulins.

Antisera.—The greater binding power of the specific globulin is even more striking in the experiments than in those of Table I, but no conclusion can be drawn from this as different sera were used ; all except 550 A flocculated very rapidly. In Experiments 29 and 30 and in Experiments 33 and 34 the equilibrium was approached from both sides, and again complete agreement was not obtained.

TABLE I.—*Binding of Phenyl-azo-p-benzene-arsonic Acid.*

	Expt. No.	Number of rabbit serum from which globulin solution was made.	Specific group of azo-protein with which rabbit was immunized.	Arsenic equivalent (millemolar).	Globulin concentration per cent. = P.	Free haptene in A. Millemolar = F.	Bound haptene in A. Millemolar = B.	B/F × P.
Controls	1	743	p-benzene-arsonic acid	Antibody precipitated	1·80	0·0200	0·0045	0·130
	2	Normal	None	0	1·60	0·0335	0·0065	0·120
	3	,,	,,	0	1·80	0·0335	0·0060	0·100
	4	726	p-benzene-sulphonic acid	Antibody not precipitated	1·80	0·0330	0·0070	0·120
	5	Normal	None	0	2·30	0·0320	0·0090	0·125
	6	743	p-benzene-arsonic acid	Antibody precipitated	2·85	0·0310	0·0115	0·130
	7	742	,, ,,	Ditto	1·50	0·1850	0·0100	0·035
	8	550 B	,, ,,	0·090	1·70	0·0110	0·0080	0·430
	9	,,	,, ,,	0·090	1·70	0·0285	0·0160	0·330
	10	,,	,, ,,	0·090	1·70	0·0275	0·0165	0·350
	11	,,	,, ,,	0·090	1·95	0·0760	0·0320	0·215
	12	,,	,, ,,	0·090	1·95	0·0730	0·0405	0·280
	13	,, diluted	,, ,,	0·045	1·00	0·0825	0·0185	0·225
	14	,, ,,	,, ,,	0·045	1·00	0·0795	0·0215	0·270
	15	722	,, ,,	0·090	2·15	0·0300	0·0135	0·205
	16	745 A	,, ,,	0·070	1·35	0·0285	0·0165	0·430
	17	745 B	,, ,,	0·210	1·50	0·0155	0·0130	0·570
	18	,,	,, ,,	0·210	1·50	0·0265	0·0200	0·500
	19	,,	,, ,,	0·210	1·50	0·0770	0·0660	0·570
	20	,,	,, ,,	0·210	1·50	0·1830	0·0690	0·250
	21	743	,, ,,	0·100	3·35	0·0265	0·0205	0·230
	22	742	,, ,,	0·210	2·00	0·0225	0·0290	0·645

TABLE II.—*Binding of Tyrosin-azo-p-benzene-arsonic Acid.*

	Expt. No.	Number of rabbit serum from which globulin solution was made.	Specific group of azo-protein with which rabbit was immunized.	Arsenic equivalent (millemolar).	Globulin concentration per cent. = P.	Free haptene in A. Millimolar = F.	Bound haptene in A. Millemolar = B.	B F × P.
Controls	23	Normal	None	0	1·90	0·0305	0·0085	0·13
	24	,,	,,	0	1·90	0·0305	0·0095	0·16
	25	396	p-benzene-arsonic acid	Antibody precipitated	2·00	0·0310	0·0075	0·11
	26	Normal	None	0	3·40	0·0300	0·0090	0·09
	27	550 A.	p-benzene-arsonic acid	0·07	2·60	0·0255	0·0195	0·75
	28	396	,, ,,	Not estimated	4·10	0·0210	0·0290	1·46
	29	396	,, ,,	0·22	2·85	0·0215	0·0265	1·24
	30	396	,, ,,	0·22	2·85	0·0180	0·0265	1·40
	31	396 diluted	,, ,,	0·10	1·40	0·0250	0·0230	0·92
	32	544	,, ,,	0·21	3·85	0·0210	0·0295	1·45
	33	544	,, ,,	0·21	3·35	0·0185	0·0325	1·75
	34	544	,, ,,	0·21	3·35	0·0145	0·0315	2·18
	35	544	,, ,,	0·21	3·50	0·0185	0·0355	1·92

TABLE III.—*Binding of Phenyl-azo-p-benzene-sulphonic Acid.*

	Expt. No.	Numbit of rabbit serum from which globulin solution was made.	Specific group of azo-protein with which rabbit was immunized.	Globulin concentration per cent. = P.	Free haptene in A. Millemolar = F.	Bound haptene in A. Millemolar = B.	B F × P.
	35	745 A.	p-benzene-arsonic acid	1·30	0·0305	0·0130	0·32
	36	722	,, ,,	1·40	0·0305	0·0125	0·29
Controls	37	Normal	None	1·55	0·0285	0·0155	0·35
	38	742	p-benzene-arsonic acid	2·00	0·0255	0·0225	0·44
	39	Normal	None	2·30	0·0255	0·0225	0·38
	40	743	p-benzene-arsonic acid	3·35	0·0250	0·0235	0·37
	41	726	p-benzene-sulphonic acid	1·30	0·0290	0·0145	0·38

Phenyl-azo-p-benzene-sulphonic Acid (Table III).

This dye was more strongly absorbed by the non-specific globulins than the two previously studied. It was equally bound by the globulin of the sera of normal rabbits and of those immunized with azo-proteins prepared with p-amino-benzene-arsonic acid. Also it was no more strongly bound by the globulin of serum 726, from a rabbit immunized with protein coupled with p-amino-benzene-sulphonic acid.

This globulin solution was a relatively weak antibody; it took half an hour to form a precipitate when mixed with the optimum proportion of test antigen, but in this respect it was no worse than 550 B, and better than 722. We have not so far been successful in obtaining strong antibody to the p-benzene-sulphonic acid antigens.

DISCUSSION.

These results give a satisfactory direct proof that the haptenes prepared from p-amino-benzene-arsonic acid combine specifically with the globulins of the homologous antiserum. They also show that the reaction is specific, since globulins from antisera to azo-proteins prepared from p-amino-benzene-arsonic acid did not bind the *p*-benzene-sulphohic acid haptenes to a greater degree than control globulins, and *vice versâ*.

The results with the p-benzene-sulphonic acid haptene are unsatisfactory. Presumably the affinity of haptene for antibody is much less in this case, and the specific combination is not detectable owing to the much greater non-specific adsorption.

The amount of protein precipitated by the optimum amount of antigen was 1 mgm. per 1 c.c. in the case of globulin solution 745 B and 2 mgm. per 1 c.c. from globulin solution 742. Even if the protein so precipitated forms only one-half of the total specific protein in these solutions, this means that the ratio $B/F \times P$ is twenty times as great for the specific antibody globulin as for the non-specific globulin used for controls. It is probable that far more satisfactory information on the combination of haptene and antibody would be obtained if it were possible to free the antibody more completely from non-specific protein, but our attempts to do so, by methods resembling those used by Felton for purifying antipneumococcus antibodies, have failed.

Taking our present results, those given by Experiments 8 to 14 (Table I) will fit the equation of a bimolecular reaction with a dissociation constant of about 1×10^{-4}. They would, however, also fit a Freundlich adsorption isotherm. But the result of Experiments 17 to 20 would not fit either type of curve.

The solutions 745 B and 742 were of the same strength as estimated by the optimal proportions methods, but 742 bound much more haptene. However, 742 flocculated in about half the time required by 745 B; also the amount of precipitate obtained at optimal proportions differed, for 1 c.c. of 742 gave 2·5 mgm. of precipitate, while 745 B gave only 1·2 mgm.

In none of our experiments has there been any evidence of precipitate formation in any of the antibody haptene mixtures, nor have we found a precipitate in mixtures of antibodies and haptenes put up in the same

proportions in special tubes for observation. The fixation of haptene in A cannot therefore be ascribed to precipitation. However, a strong fresh serum was found to give a very slight precipitate on standing over-night with 0·036 m. M. tyrosin-azo-p-benzene-arsonate, but not with the same strength of phenyl-azo-p-benzene arsonate.

It is possible that, for the formation of a precipitate, it may be necessary that more than one specific group should be attached to one haptene or antigen molecule. Such groups when they combine with the receptors on the antibody molecule may strain it, and cause some intramolecular rearrangement leading to precipitation. This would explain the absence of precipitation with the phenol derivative, slight precipitation with tyrosin and resorcin derivatives (as found by Landsteiner and Van der Scheer, 1932), in which two specific groups are coupled to the molecule, and full precipitation with azo-proteins containing many specific groups per molecule.

SUMMARY.

The specific combination of the globulins of the sera of rabbits, immunized with azo-proteins made from p-amino-benzene-arsonic acid, with haptenes made by coupling this compound with phenol or tyrosin, has been demonstrated by a direct method.

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